(54) Title: CLONED GLUTAMIC ACID DECARBOXYLASE

(57) Abstract

Isolated polypeptides useful in ameliorating GAD-associated autoimmune disease as well as diagnostic and therapeutic methods of using the peptides are disclosed.

- 1 -

CL NED GLUTAMIC ACID DECARBOXYLASE

This application is a continuation-in-part
application of U.S. Serial No. 07/716,909, filed June
18, 1991 which is a continuation-in-part application of
U.S. Serial No. 07/586,536, filed September 21, 1990.

BACKGROUND OF THE INVENTION

1. FIELD OF THE INVENTION

The present invention relates to glutamic acid
decarboxylase$_{65}$ (GAD$_{65}$) polypeptides and methods of using
GAD$_{65}$ polypeptides diagnostically and therapeutically in
autoimmune disease.

2. DESCRIPTION OF THE BACKGROUND ART

Insulin-dependent diabetes mellitus (IDDM; type I
diabetes) is one of the most common metabolic
disorders. In the United States, IDDM affects
approximately one in 300 to 400 people, and
epidemiological studies suggest that its incidence is
increasing. The disease results from the autoimmune
destruction of the insulin-producing β-cells of the
pancreas. More specifically, the preonset stage is
characterized by "insulitis", in which lymphocytes
infiltrate the pancreatic islets and selectively
destroy the β-cells. Insulitis may be present for many
years before the onset of clinical symptoms. The
typical IDDM presentation of hyperglycemia appears only
after at least 80% of the insulin-producing β-cells are
lost. The remaining β-cells are destroyed during the
next few years.

Although insulin therapy allows most IDDM patients
to 1 ad normal lives, this replacement is imp rfect and
do s not completely restore metabolic homeostasis.
Thus, severe complications which result in dysfunctions

- 2 -

of the eye, kidney, heart, and other organs are common
in IDDM patients undergoing insulin therapy. Because
of this, it is highly desirable to extend the latency
period and prevent progression (e.g., through
5   administration of immunosuppressant drugs to interfere
with the autoimmune process and insulin to achieve
better control of the effects of sustained
hypoglycemia) between the start of β-cell destruction
and the actual requirement of insulin replacement
10   (i.e., when 80% of the β-cells are destroyed).
Therefore, a diagnostic test which determines the
beginning of β-cell destruction would allow the
clinician to administer immunosuppressant drugs
(Silverstein, et al., New England Journal of Medicine,
15   319:599-604, 1988) or prophylactic insulin therapy
(Keller, et al., Lancet, 341:927, 1993) to extend this
latency period and thus significantly delay the onset
of insulin replacement side effects.

        Many IDDM patients have sera which contain
20   antibodies to a 64kD molecule (Baekkeskov, et al.,
J.Clin.Invest., 79:926-934, 1987; Atkinson, et al.,
Lancet, 335:1357-1360, 1990), to islet cell cytoplasmic
(ICA) molecules or islet cell surface (ICSA) molecules
(Bottazzo, et al, Lancet, 1:668-672, 1980), or to
25   insulin (Palmer, et al., Science, 222:1137-1139, 1983;
Atkinson, et al., Diabetes, 35:894-898, 1986).
Atkinson and coworkers (Atkinson, et al., Lancet,
335:1357-1360, 1990) have demonstrated that the
presence of antibodies to the 64kD molecule in human
30   sera appears to be the earliest and most reliable
indicator that onset of IDDM symptoms will eventually
occur.

        Recently, Baekkeskov and coworkers established that
the 64kD molecule and glutamic acid decarboxylase (GAD)
35   have s veral antigenic epitopes in common and thus they
may be identical or very similar molecul s. Although
this identification is an important finding, the use of

- 3 -

this information as a diagnostic tool for predicting
IDDM is quite cumbersome and limited unless knowledge
of the molecular biology of GAD is known. Studies by
Kaufman, et al., (J. Clin. Invest., 89:283, 1992)
5   established that the 64kD molecule was intact GAD_{65}.
Consequently, the cloning and subsequent production of
large quantities of GAD_{65} or a GAD molecule which is
antigenically substantially identical to the GAD_{65}
molecule or fragments of the GAD_{65} molecule, both of
10   which can be easily purified, will allow the
development of a diagnostic kit designed to predict
IDDM as well as effective therapeutic modalities. The
present invention provides a means for accomplishing
these results.

15   **SUMMARY OF THE INVENTION**
          The present invention arose out of the discovery
that recombinant DNA technology could be used to
produce eukaryotic GAD_{65} polypeptide and that GAD_{65}
polypeptide could be used in the diagnosis and therapy
20   of patients with autoimmune disease. Particularly
relevant is the use of eukaryotic GAD_{65} polypeptide in
the diagnosis and therapy of patients having, or at
risk of having, GAD-associated autoimmune disorders
such as insulin-dependent diabetes mellitus (IDDM) or
25   stiff man disease.
          A major advantage of the present invention is that
it provides the art with a ready source of eukaryotic
GAD_{65} polypeptide corresponding to that purified from
natural sources, while avoiding the problems associated
30   with the isolation of naturally occurring eukaryotic
GAD_{65} polypeptide when separating it from other
eukaryotic non-GAD_{65} polypeptides. This absence of
other eukaryotic non-GAD_{65} polypeptides is significant
in that it allows the development of test systems which
35   will nly det ct antibodies specifically reactive with
GAD_{65} polypeptides.

- 4 -

Another advantage of providing eukaryotic GAD_{65} polypeptide in host cells is that by so doing, it is possible to obtain much larger quantities of the polypeptide than are currently practicably available

5   from natural sources. As a consequence, not only is it possible to use the polypeptide of the invention to more accurately classify and treat patients with such autoimmune diseases as IDDM, but it is also now possible to provide commercially useful quantities of

10 GAD polypeptide for use in diagnostic systems and pharmaceutical compositions.

DESCRIPTION OF THE DRAWINGS

FIGURE 1   Cloning strategy for obtaining GAD_{65} and GAD_{67} specific cDNA probes.

15   FIGURE 2   DNA sequence and corresponding amino acid sequence for rat GAD_{65}.

FIGURE 3   DNA sequence and corresponding amino acid sequence for human GAD_{65}.

FIGURE 4   Comparison of rat GAD_{65} and human GAD_{65}

20 amino acid sequences.

FIGURE 5 GAD_{65} and GAD_{67} cDNAs hybridize to different size RNAs.

FIGURE 6   Southern blots hybridized with CDNA probes specific for GAD_{65} and GAD_{67}.

25   FIGURE 7   Immunological identification of GAD_{65} and GAD_{67}.

FIGURE 8   Proliferative T-cell responses of NOD mice to β cell antigens.

FIGURE 9   Characterization of GAD specific T-cell

30 response of NOD mice as primed Th1 cells by enhanced clonal size (a) and cell surface markers (b) and $IFN\gamma$ secretion.

FIGURE 10   Intramolecular spreading of T-cell autoimmunity within the GAD molecule.

35   FIGURE 11   Delay of onset of IDDM following immunization with GAD_{65}.

- 5 -

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the manipulation
of genetic materials by recombinant DNA procedures
which make possible the production of polypeptides
5   possessing part or all of the primary structural
conformation for one or more of the epitopes for
binding autoantibodies to glutamic acid decarboxylase$_{65}$
(GAD$_{65}$) and for polypeptides that bind to MHC receptors
to block T-cell recognition. These polypeptides are
10   highly useful for the immunological detection of
autoantibodies reactive with them, since such
autoantibodies are pre-diagnostic and indicative of
autoimmune diseases such as insulin dependent diabetes
mellitus and "stiff man" syndrome. These polypeptides
15   can also be used for purposes of screening drugs, such
as those that alter GAD function, and for generation of
polyclonal and monoclonal antibodies which, in turn,
can be used diagnostically to detect GAD$_{65}$.

The development of specific DNA sequences encoding
20   eukaryotic GAD$_{65}$ polypeptide for splicing into DNA
vectors can be accomplished using a variety of
techniques. For example, alternative methods which can
be employed include (1) the isolation of a double
stranded DNA sequence from the genomic DNA of the
25   eukaryote; (2) the chemical manufacture of a DNA
sequence to provide the necessary codons for the
polypeptide of interest; and (3) the in vitro synthesis
of a double stranded DNA sequence by reverse
transcription of mRNA isolated from a eukaryotic donor
30   cell. In the latter case, a double stranded DNA
complement of MRNA is eventually formed which is
generally referred to as CDNA.

The manufacture of DNA sequences is frequently the
method of choice when the entire sequence of amino acid
35   residues of the desired polypeptide product is known.
When th entire sequenc of amino acid residues of the
d sired polyp ptid is not known, the direct
manufacture of DNA sequences is not possible and the
method of choice is the formation of cDNA sequences.

- 6 -

Among the standard procedures for isolating cDNA
sequences of interest is the formation of plasmid-
carrying cDNA libraries which are derived from reverse
transcription of mRNA which is abundant in donor cells
5   that have a high level of genetic expression. When
used in combination with polymerase chain reaction
technology, even rare expression products can be
cloned. In those cases where significant portions of
the amino acid sequence of the polypeptide are known,
10   the production of labeled single or double stranded DNA
or RNA probe sequences duplicating a sequence
putatively present in the target cDNA may be employed
in DNA/DNA hybridization procedures which are carried
out on cloned copies of the cDNA which have been
15   denatured into a single stranded form (Jay, et al.,
Nucleic Acid Research, 11:2325, 1983).

Hybridization procedures are useful for the
screening of recombinant clones by using labeled mixed
synthetic oligonucleotide probes wherein each is
20   potentially the complete complement of a specific DNA
sequence in the hybridization sample which includes a
heterogeneous mixture of denatured double stranded DNA.
For such screening, hybridization is preferably
performed on either single stranded DNA or denatured
25   double stranded DNA. These procedures are particularly
useful in the detection of cDNA clones derived from
sources where an extremely low amount of mRNA sequences
relating to the polypeptide of interest are present.
.In other words, by using stringent hybridization
30   conditions directed toward avoidance of non-specific
binding, it is possible, for example, to allow the
autoradiographic visualization of a specific cDNA clone
by the hybridization of the target DNA to that single
probe in the mixture which is its complete complement
35   (Wallac , et al., Nucleic Acid Research, 9:879, 1981).

In addition, a GAD cDNA library can be screened by
injecting the various cDNAs into oocytes, allowing
suffici nt time for expression of th cDNA gene
products to occur, and testing for the pr senc of the

- 7 -

desired cDNA expression product, for example, by using
antibody specific for GAD$_{65}$ polypeptide, by using
functional assays for GAD$_{65}$ enzymatic activity, or by
measuring the ability of the expression product to

5   stimulate pathogenic T-cells.

Alternatively, a cDNA library can be screened
indirectly for GAD$_{65}$ peptides having at least one
epitope using antibodies to GAD$_{65}$ (Chang and Gottlieb,
J.Neurosci., 8:2123, 1988). Such antibodies can be

10   either polyclonally or monoclonally derived and used to
detect expression product indicative of the presence of
GAD$_{65}$ cDNA. Preferred are antibodies directed to an
epitope found in the first 100 amino acids of the N-
terminal portion of GAD$_{65}$.

15   Of the three above-noted methods for developing
specific DNA sequences for use in recombinant
procedures, the use of genomic DNA isolates, is the
least common. This is especially true when it is
desirable to obtain the microbial expression of

20   mammalian polypeptides because of the presence of
introns.

The present invention provides novel polypeptides
of GAD$_{65}$ which have part or all of the primary
structural conformation, that is, a continuous sequence

25   of amino acid residues, having at least one epitope for
antibodies to GAD$_{65}$ or at least one determinant for T-
cell recognition. It is possible to use the
polypeptide fragments of the invention rather than
intact GAD to detect autoantibodies to GAD. The term

30   "polypeptide," as applied to GAD polypeptide, includes
any sequence of amino acids having an epitope for
autoantibodies to GAD or binds to a T-cell MHC
receptor. Thus, the polypeptide fragments of GAD
encompassed by the invention possess a biological

35   activity such as the ability to induce and/or bind
autoantibodies to GAD, bind to T-cell MHC receptors
(specially receptors n pathogenic T-cells) and the
like.

- 8 -

The polypeptides resulting from microbial
expr ssion of the DNA sequences of the invention or
from other synthetic techniques, such as solid-phase
peptide synthesis, can be further characterized by
5   their freedom from association with other eukaryotic
polypeptides or other contaminants which might
otherwise be associated with GAD in its natural
cellular environment or in such extracellular fluids as
plasma or urine.
10   Studies by the present inventors unequivocally
establish that GAD_{65} and GAD_{67} are encoded by distinct
genes and are not produced, for example, by
post-transcriptional or post-translational modification
of a common genomic sequence. Evidence proving that
15   GAD_{65} and GAD_{67} are encoded by different genes include:
(a) the largest contiguous sequence of exact identity
between GAD_{65} and GAD_{67} cDNAs is only 17 nucleotides in
length, (b) cDNAs from GAD_{65} and GAD_{67} do not cross
hybridize with each other's or with each other's mRNA
20   under low stringency conditions (2.0 x SSC, 0.01% SDS,
23°C), and (c) GAD_{65} and GAD_{67} cDNAs do not cross
hybridize with isolated genomic clones encoding GAD_{67}
and GAD_{65}, respectively.
The term "host" includes not only prokaryotes, but
25   also such eukaryotes as yeast, filamentous fungi, plant
and animal cells, as well as insect cells which can
replicate and express an intron-free DNA sequence of
eukaryotic GAD_{65}. However, prokaryotes are preferred as
the host organism for screening purposes while
30   eukaryotic cells, especially insect cells, are
preferred for expression.
The term "prokaryotes" includes all bacteria which
can be transformed or transfected with the gene for the
expression of GAD_{65}. Prokaryotic hosts may include gram
35   negative as well as gram positive bacteria such as, f r
example, *E. coli, S. typhimurium, Serratia marcescens*
and Bacillus subtilis.

- 9 -

A recombinant DNA molecule coding for the GAD_{65} polypeptides can be used t transform or transfect the host using any of the techniques commonly known to those of ordinary skill in the art. Especially
5   preferred is the use of a plasmid or a virus containing the GAD_{65} coding sequence for purposes of prokaryotic transformation or transfection, respectively. Alternatively, liposomes containing the DNA of interest can be used to obtain expression in the host (Zhu, et
10   al., *Science*, 261:209, 1993)

Methods for preparing fused, operably linked genes and expressing them in bacteria are well-known in the art (Maniatis, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring
15   Harbor, NY, 1989). The genetic constructs and methods described therein can be utilized for expression of GAD_{65} in prokaryotic hosts.

In general, expression vectors containing promotor sequences which facilitate the efficient transcription
20   of the inserted eukaryotic genetic sequence are used in connection with the host. The expression vector typically contains an origin of replication, a promoter, and a terminator, as well as specific genes which are capable of providing phenotypic selection of
25   the transformed cells. The transformed prokaryotic hosts can be grown in fermentors and cultured according to techniques known in the art to achieve optimal cell growth. The polypeptides of the invention can then be isolated from the grown medium, cellular lysates, or
30   cellular membrane fractions.

The isolation and purification of the expressed polypeptides of the invention may be by any conventional means such as, for example, preparative chromatographic separations and immunological
35   separations such as those involving the us of m noclonal or polyclonal antibody.

By having provided the sequence of amino acid residu s f GAD_{65}, the present invention provides for the manufactur of DNA s quences which code for the

- 10 -

host expression of polypeptide analogs or derivatives
of GAD$_{65}$ which diff r from naturally-occurring forms in
terms of the identity or location of one or more amino
acid residues and which share some or all of the
5   epitopes of naturally-occurring polypeptide forms.

The novel DNA sequences of the invention include
all sequences useful in providing the expression in
prokaryotic or eukaryotic host cells of polypeptides
which have at least a part of the primary structural
10   conformation for one or more epitopes capable of
reacting with autoantibodies to GAD$_{65}$ which are
comprehended by: (a) the DNA sequence as set forth in
Figures 2 or 3 or their complementary strands; (b) DNA
sequences which hybridize to DNA sequences defined in
15   (a) or fragments thereof; and (c) DNA sequences which,
but for the degeneracy of the genetic code, would
hybridize to DNA sequences defined in (a) and (b)
above. Specifically comprehended in (b) are genomic
DNA sequences which encode allelic variant forms of
20   GAD$_{65}$. Part (c) specifically comprehends the
manufacture of DNA sequences which encode GAD$_{65}$, GAD$_{65}$
fragments, and GAD$_{65}$ analogs wherein the DNA sequences
thereof may incorporate codons which facilitate
translation of mRNA in non-vertebrate hosts.

25   Since the cDNA sequence of the invention encodes
essentially the entire human or rat GAD$_{65}$ molecule, it
is now a matter of routine to prepare, subclone, and
express smaller polypeptide fragments of cDNA from this
or a corresponding cDNA sequence which would encode as
30   few as one epitope for autoantibodies to human or rat
GAD$_{65}$. The presence of such an epitope on a cloned
polypeptide can then be confirmed using, for example,
serum from a patient with autoantibodies to GAD$_{65}$. An
example of such a smaller peptide is the first
35   approximately 100 amino acids from the N-terminus f
GAD$_{65}$ (shown in Figure 3). This amino acid sequence is
ssentially absent from GAD$_{67}$. Other xamples of
specific peptides of the invention are shown in Table 7

- 11 -

as well as the approximate carboxy-terminal two-thirds
of GAD from about amino acid 224 to about amino acid
585. Especially preferred in the carboxy-terminal two-
thirds of GAD is the amino acid segment from about
5   amino acid 224 to about amino acid 398.

The present invention further relates to monoclonal
antibodies which are specific for the polypeptides of
the invention as well as the diagnostic and therapeutic
use of these monoclonal antibodies. This specificity
10   enables the monoclonal antibody, and like monoclonal
antibodies with like specificity, to be used to bind
the polypeptide of the invention when the polypeptide,
or amino acids comprising the polypeptide, are present
in specimens or a host, such as a human.

15   Numerous techniques can be utilized to produce the
monoclonal antibodies of the invention without
resorting to undue experimentation. To a great extent,
the products of such monoclonal antibodies is rendered
routine because of the highly defined nature of the
20   polypeptides of the invention. Thus, whether the
polypeptides of the invention are used for immunization
and/or screening, the very limited number of
immunogenic determinants on the polypeptides greatly
simplifies the identification of cell lines producing
25   monoclonal antibodies of the invention, for example, by
limiting the repertoire of clonal expression possible.

One very useful type of cell line for expression of
the monoclonal antibodies of the invention is the
hybridoma. The general method used for production of
30   hybridomas producing monoclonal antibody is well known
(Kohler and Milstein, *Nature*, 256:495, 1975). The
resulting hybridomas were then screened for production
of monoclonal antibodies capable of binding to the
polypeptides of the invention.

35   Th techniques of sensitization and/or
immunization, cell fusion, ascites production,
selecti n f mixed hybridomas, or subcloning of
monoclonal hybridomas are generally well known in the
art. Attention is directed to Koprowski, *et al.*, U.S.

- 12 -

Patent No. 4,172,124, Koprowski, *et al.*, U.S. Patent
No. 4,196,265, or Douillard, J.Y. and Hoffman, T.,
Basic Facts about Hybridomas, in *Compendium of
Immunology*, Vol. II, L. Schwartz, ed. (1981), which are
5   herein incorporated by reference. In general, the
purified peptides can be modified to have a cystine
attached at the C-terminus to permit unidirectional
attachment of the synthetic peptide to an immunogenic
protein through a connecting bridge, for example,
10   maleimidobenzoylated (MB)-keyhole limpet hemocyanin
(KLH). Other immunogenic conjugates can also be used,
for example, albumin, and the like. The resulting
structure may have several peptide structures linked to
one molecule of protein.
15   Somatic cells derived from a host immunized against
the synthetic peptides can be obtained by any suitable
immunization technique. The host subject is immunized
by administering the antigen, usually in the form of a
protein conjugate, as indicated above, by any suitable
20   method, preferably by injection, either
intraperitoneally, intravenously, subcutaneously, or by
intra-foot pad. Adjuvants may be included in the
immunization protocol.
          The initial immunization with the protein bound
25   antigen can be followed by several booster injections
given periodically at intervals of several weeks. The
antibody contained in the plasma of each host can then
be tested for its reactivity with the immunizing
polypeptide of the invention. The host having the
30   highest response is usually most desirable as the donor
of the antibody secreting somatic cells used in the
production of hybridomas. Alternatively,
hyperimmunization can be effected by repeatedly
injecting additional amounts of peptide-protein
35   conjugate by intravenous and/or intrap ritoneal route.

- 13 -

The isolation of hybridomas producing monoclonal antibodies of the invention can be accomplished using routine screening techniques which permit determination of the elementary reaction pattern of the monoclonal antibody of interest. Thus, if a monoclonal antibody being tested binds with a polypeptide of the invention, then the antibody being tested and the antibody produced by the hybridomas of the invention are equivalent.

Alternatively, since the invention teaches polypeptides or amino acid sequences which are specifically required for binding of the preferred monoclonal antibodies of the invention, it is now possible to use these peptides for purposes of immunization to produce hybridomas which, in turn, produce monoclonal antibodies specific for the polypeptide. This approach has the added advantage of decreasing the repertoire of monoclonal antibodies generated by limiting the number of antigenic determinants presented at immunization by the polypeptide. The monoclonal antibodies produced by this method can be screened for specificity using standard techniques, for example, by binding polypeptide to a microtiter plate and measuring binding of the monoclonal antibody by an ELISA assay.

It is also possible to determine, without undue experimentation, if a monoclonal antibody has the same specificity as a monoclonal antibody of the invention by ascertaining whether the former prevents the latter from binding the polypeptide of the invention. If the monoclonal antibody being tested competes with the monoclonal antibody of the invention, as shown by a decrease in binding by the monoclonal antibody of the invention, then it is likely that the two monoclonal antibodi s bind to the same, or a closely related, epitope.

Still anoth r way t d termine whether a monoclonal antibody has the sp cificity of a monoclonal antibody of the invention is to pre-incubate th monoclonal

- 14 -

antibody of the invention with the polypeptide of the
invention with which it is normally reactive, and then
add the monoclonal antibody being tested to determine
if the monoclonal antibody being tested is inhibited in
5   its ability to bind the antigen. If the monoclonal
antibody being tested is inhibited then, in all
likelihood, it has the same, or a closely related,
epitopic specificity as the monoclonal antibody of the
invention.

10   The GAD_{65} of the invention is particularly suited
for use in immunoassays in which it can be utilized in
liquid phase or bound to a solid phase carrier. In
addition, GAD_{65} used in these assays can be detectably
labeled in various ways.

15   Examples of immunoassays which can utilize the GAD_{65}
of the invention are competitive and non-competitive
immunoassays in either a direct or indirect format.
Examples of such immunoassays are the radioimmunoassay
(RIA), the sandwich (immunometric assay) and the
20   Western blot assay. Detection of antibodies which bind
to the GAD_{65} of the invention can be done utilizing
immunoassays which run in either the forward, reverse,
or simultaneous modes, including immunohistochemical
assays on physiological samples. The concentration of
25   GAD_{65} which is used will vary depending on the type of
immunoassay and nature of the detectable label which is
used. However, regardless of the type of immunoassay
which is used, the concentration of GAD_{65} utilized can
be readily determined by one of ordinary skill in the
30   art using routine experimentation.

     The GAD and GAD fragments of the invention can be
bound to many different carriers and used to detect the
presence of antibody specifically reactive with the
polypeptide. Alternatively, the carrier-bound GAD and
35   GAD fragments can be used therapeutically for
extracorporeal absorption of autoimmune antibodies in
patients having, or at risk of having, GAD-associated
disorders. Examples of well-known carriers include
glass, polystyr ne, polyvinyl chloride, polypropylene,

- 15 -

polyethylene, polycarbonate, dextran, nylon, amyloses, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble or insoluble for purposes of the

5   invention. Those skilled in the art will know of other suitable carriers for binding GAD_{65}, or will be able to ascertain such, using routine experimentation.

There are many different labels and methods of labeling known to those of ordinary skill in the art.

10   Examples of the types of labels which can be used in the present invention include enzymes, radioisotopes, colloidal metals, fluorescent compounds, chemiluminescent compounds, and bioluminescent compounds.

15   Alternatively, the polypeptide of the invention which comprises the GAD enzymatic domain can be used to detect antibodies to GAD by measuring GAD enzymatic activity. For example, GAD_{65} and a specimen suspected of having antibodies to GAD_{65} can be incubated for a

20   period of time and under conditions sufficient to allow binding to occur between GAD_{65} and the antibodies. The reaction product is precipitated and then tested for GAD enzymatic activity.

For purposes of the invention, the antibody which

25   binds to GAD_{65} of the invention may be present in various biological fluids and tissues. Any sample containing a detectable amount of antibodies to GAD_{65} can be used. Normally, a sample is a liquid such as urine, saliva, cerebrospinal fluid, blood, serum and

30   the like, or a solid or semi-solid such as tissue, feces and the like.

The materials for use in the assay of the invention are ideally suited for the preparation of a kit. Such a kit may comprise a carrier means being

35   compartmentalized to receive in close confin ment one or mor container means such as vials, tubes and the like, each of the container means comprising on of th separat elements to be used in the method. For exampl , one of the container means may compris GAD_{65}

- 16 -

bound to a carrier. A second container may comprise
soluble, detectably-labeled second antibody, in
lyophilized form or in solution.

In addition, the carrier means may also contain a
5   plurality of containers each of which comprises
different, predetermined amounts of GAD_{65}. These latter
containers can then be used to prepare a standard curve
into which can be interpolated the results obtained
from the sample containing the unknown amount of
10   autoantibodies to GAD_{65}.

In using the kit all the user has to do is add, to
a container, a premeasured amount of a sample
containing a measurable, yet unknown amount of
autoantibodies to GAD_{65} to be detected, a premeasured
15   amount of carrier-bound GAD_{65} present in the first
container, and a premeasured amount of the detectably
labeled second antibody present in the second
container. Alternatively, the non-detectably labeled
GAD_{65} can be provided attached to the container to which
20   the sample and the detectably labeled second antibody
are added. After an appropriate time for incubation,
an immune complex is formed and is separated from the
supernatant fluid, and the immune complex or the
supernatant fluid are detected, as by radioactive
25   counting or addition of an enzyme substrate, and color
development.

In an alternative embodiment, a kit comprising the
GAD polypeptide of the invention can be used to detect
·the stage of GAD-associated autoimmune disease in a
30   patient. As further shown herein, Applicants have
discovered that certain GAD peptides or fragments are
associated with different levels of progression in the
autoimmune disease and that the level of disease
process can be ascertained by looking at immune cell
35   proliferative response, such as that of the pathogenic
T-cell of th pati nt.

The term "ameliorate" denotes a lessening of th
detrimental effect of the autoimmune response in the
patient receiving therapy. The term "therapeutically

- 17 -

effective" means that the amount of GAD_{65} polypeptid
used is of sufficient quantity to ameliorate the cause
of disease due to the autoimmune response.

The GAD_{65} polypeptides, including whole GAD_{65}, of the
5   invention can be used therapeutically in patients
having, or at risk of having, an autoimmune response
associated with GAD_{65}. Such therapy can be
accomplished, for example, by the administration of
GAD_{65} polypeptide to induce tolerance to GAD. Such
10   administration can utilize unlabeled as well as labeled
GAD_{65} polypeptide. When unlabeled GAD_{65} polypeptide is
utilized advantageously, it would be in a form wherein,
for example, the GAD_{65} polypeptides are in fragments
which are too small to stimulate an immune response,
15   but large enough to bind, or block, the continuance of
the autoimmune response. For example, GAD_{65} could be
digested enzymatically into epitope-sized peptides
(typically 5-12 amino acids in length) and thereby bind
to Fab binding portions present in the body fluids, or
20   on the surface of immune cells, of the patient with
autoimmune disease. Alternatively, peptides having at
least one determinant for binding to T-cell MHC
receptor can be similarly produced or chemically
synthesized.

25   Alternatively, the GAD_{65} polypeptides of the
invention can be administered labeled with a
therapeutic agent. These agents can be coupled either
directly or indirectly to the GAD_{65} polypeptides of the
invention. One example of indirect coupling is by use
30   of a spacer moiety. These spacer moieties, in turn,
can be either insoluble or soluble (Diener, et al.,
Science, 231:148, 1986) and can be selected to enable
drug release from the GAD_{65} polypeptide at the target
sit . Examples of therapeutic agents which can be
35   coupl d to the GAD_{65} polypeptides of the inventi n for
immunotherapy are drugs, radioisotopes, lectins, and
toxins.

- 18 -

The drugs with which can be conjugated to the GAD_{65} polypeptides of the invention include comp unds which are classically referred to as drugs such as mitomycin C, daunorubicin, and vinblastine.

In using radioisotopically conjugated GAD_{65} polypeptides of the invention for immunotherapy, certain isotopes may be more preferable than others depending on such factors as leukocyte distribution as well as stability and emission. Depending on the autoimmune response, some emitters may be preferable to others. In general, α and β particle-emitting radioisotopes are preferred in immunotherapy. Preferred are short range, high energy α emitters such as ^{212}Bi. Examples of radioisotopes which can be bound to the GAD_{65} polypeptides of the invention for therapeutic purposes are ^{125}I, ^{131}I, ^{90}Y, ^{67}Cu, ^{212}Bi, ^{211}At, ^{212}Pb, ^{47}Sc, ^{109}Pd and ^{188}Re.

Lectins are proteins, usually isolated from plant material, which bind to specific sugar moieties. Many lectins are also able to agglutinate cells and stimulate lymphocytes. However, ricin is a toxic lectin which has been used immunotherapeutically. This is accomplished by binding the α-peptide chain of ricin, which is responsible for toxicity, to the antibody molecule to enable site specific delivery of the toxic effect.

Toxins are poisonous substances produced by plants, animals, or microorganisms that, in sufficient dose, are often lethal. Diphtheria toxin is a substance produced by *Corynebacterium diphtheria* which can be used therapeutically. This toxin consists of an α and β subunit which under proper conditions can be separated. The toxic A component can be bound to GAD_{65} polypeptide and used for site specific delivery to a leukocyte expressing a receptor for GAD_{65} polypeptide.

Other therapeutic agents which can be c upled to the GAD_{65} polypeptides of the invention, as well as *ex vivo* and *in vivo* therapeutic protocols, are known, or

- 19 -

can be easily ascertained, by those of ordinary skill
in the art.

The present invention also relates to a polypeptide
which can be administered therapeutically to

5   ameliorate, or utilized diagnostically to identify, the
disease process in patients having, or at risk of
having, this disease. The conventional single-letter
code used to represent the various amino acids relates
as follows:

10   **TABLE 1**

Phe: F   Leu: L   Ile: I Met: M

Val: V   Ser: S   Pro: P Thr: T

Ala: A   Tyr: Y   His: H Gln: Q

15   Asn: N   Lys: K   Asp: D Glu: E

Cys: C   Trp: W   Arg: R Gly: G

A polypeptide sequence of the invention was
identified by comparing the amino acid sequences of

20   human GAD_{65}, human GAD_{67}, and the P2-C protein of the
picornavirus, coxsackie virus. The P2-C polynucleotide
plays a role in the virus membrane bound replication
complex. These analyses established the presence of an
extensive sequence similarity between both GAD_{65}

25   molecules and the coxsackie virus. A core polypeptide
of six contiguous amino acid residues of the GAD_{65} and
P2-C polypeptide are identical in amino acid sequence.
Indeed, of the 24 amino acids in the polypeptide, 19
·are identical or conserved. In addition, there also

30   exists a high charge density and the presence of a
proline residue which would render this region highly
antigenic (see Table 2).

TABLE 2

COMPARISON OF AMINO ACID SEQUENCES

Protein	Amino Acid Sequence
Human GAD_{67}	$_{258}$S I M A A R Y K Y F P E V K T K G M A A V P K L$_{281}$
Human GAD_{65}	$_{250}$A M M I A R F K M F P E V K E K G M A A L P R L$_{273}$
Coxsackie Virus P2-C	$_{28}$F I E W L K V K I L P E V K E K H E F - L S R L$_{50}$

The solid line encloses identical amino acids whereas the dashed line encloses amino acid residues with similar charge, polarity, or hydrophobicity.

- 21 -

In Table 2, the solid line encloses identical amino acids whereas the dashed line encloses amino acid residues with similar charge, polarity, or hydrophobicity.

5   The discovery of this common polypeptide region supports an etiologic role for "molecular mimicry" in the precipitation of diabetes. Thus, where a patient genetically susceptible to IDDM is infected by a coxsackie virus, the immune response to the similar GAD

10   sequence in the patient's β-cells. The immunological response is maintained by the antigenically similar GAD polypeptides resulting in the eventual destruction of the β-cells and the subsequent presentation of IDDM.

At present, it is believed that the destruction of

15   pancreatic β-cells in IDDM is mediated by a cellular autoimmune response. As described herein, a polypeptide of the invention can ameliorate the autoimmune response to GAD. Because of the complexity of autoimmune disease, it is possible to envision

20   numerous possible therapeutic modalities which would allow the polypeptides of the invention to be used to ameliorate such diseases. In one embodiment, it appears that the polypeptides of the invention can be utilized to block recognition by a specific T cell

25   receptor (TCR) or an MHC receptor presenting an autoimmune antigen on the surface of an antigen presenting cell (APC). The inhibition of such recognition might occur, for example, by providing the patient with the polypeptide of the invention which, in

30   turn, can displace the autoimmune antigen being presented in the antigen-cleft of the MHC receptor. However, although not wanting to be bound to a particular theory, it is believed that the polypeptides of the invention probably act to induce or restore a

35   tolerogenic stat by direct interaction with the appropriate TCR on the surface of a GAD specific pathogenic T-cell. This latter therap utic appr ach of

- 22 -

direct interaction with the TCR is supported by the
examples and suggests that suppression of the
autoimmune response can be achieved through induction
of high-zone tolerance by use of high concentrations of
5   polypeptide, preferably soluble. Another possible
mechanism is that the polypeptide of the invention may
play a role in anergizing pathogenic T cells by binding
to the T cell MHC receptor, thereby preventing the
appropriate costimulatory signal.

10   Alternatively, the polypeptides of the invention
could be used to stimulate a T-suppressor cell
population in order to restore self-recognition and,
thereby, ameliorate the autoimmune disease.
Stimulation of T-suppressor cell populations could be
15   achieved, for example, by use of a bi-specific antibody
having one variable region specific for an epitope
present on the autoimmune antigen residing in the cleft
of the MHCII receptor and, a second variable region
specific for an epitope present on the CD8$^+$ receptor.
20   The production of antibody specific for the polypeptide
of the invention is a matter of routine to those of
skill in the art, as is the preparation of bi-specific
antibodies having specificity for 2 or more epitopes.

          Polypeptide analogs of the present invention may be
25   designed which will compete for recognition of self-
antigens at the level of antigen presentation or induce
anergy in T cells, due to a lack of a costimulatory
signal. Since MHC molecules contain a single peptide
binding site, it is possible to design polypeptides
30   which will bind with high affinity to disease-
associated MHC molecules, but will not activate
disease-causing T-helper cells. Such polypeptides act
as antagonists for self-antigen recognition. In the
present invention, support for this mechanism is found
35   in the examples, especially Example 7. Precedent for
such an approach arises from observation that a mouse
lysozyme polyp ptide, its lf non-immunogenic, can

- 23 -

compete for MHC binding with an immunogenic polypeptide
from hen-egg white lysozym and thereby reduce T cell
activation by that polypeptide (Adorini, et al.,
Nature, 334:623-625, 1988) as well as studies using T-
5   cell receptor peptides to block formation of complex
between T-cells, autoantigen and MHC (Howell, et al.,
Science, 246:668, 1989). Similarly, such a therapeutic
approach for screening effective polypeptide analogs
has been utilized in such autoimmune diseases as
10   experimental autoimmune encephalomyelitis (EAE)
(Wraith, et al., Cell, 59:248, 1989; Urban, et al.,
Cell, 59:257, 1989).

The single-letter symbols used to represent the
amino acid residues in the polypeptides of the present
15   invention are those symbols commonly used in the art.
The peptides of the invention include not only the
natural amino acid sequences, but also peptides which
are analogs, chemical derivatives, or salts thereof.
The term "analog" or "conservative variation" refers to
20   any polypeptide having a substantially identical amino
acid sequence to a polypeptide provided herein and in
which one or more amino acids have been substituted
with chemically similar amino acids. For example, one
polar amino acid, such as glycine or serine, may be
25   substituted for another polar amino acid; or one acidic
amino acid, such as aspartic acid may be substituted
for another acidic amino acid, such as glutamic acid;
or a basic amino acid, such as lysine, arginine, or
histidine may be substituted for another basic amino
30   acid; or a non-polar amino acid such as alanine,
leucine, or isoleucine may be substituted for another
non-polar amino acid.

The term "analog" or "conservative variation" also
means any polypeptide which has one or more amino acids
35   deleted from or added to a polypeptide of the pr sent
invention, but which still retains a substantial amino
acid sequence homology to such peptide. A substantial

- 24 -

sequence homology is any homology greater than 70%,
preferably at least about 80%, and more preferably at
least about 90%. The term "fragment" also means any
shorter version of the polypeptides identified herein
5   having at least 6 amino acid residues, wherein the
fragment possesses biological activity, or is a
fragment capable of inhibiting the stimulation of T-
cells by a stimulating polypeptide fragment or
substantially full-length molecule.

10   The term "chemical derivative" means any
polypeptide derived from a polypeptide of the present
invention and in which one or more amino acids have
been chemically derivatized by reaction of the
functional side groups of amino acid residues present
15.   in the polypeptide. Thus, a "chemical derivative" is a
polypeptide that is derived from the sequences or
polypeptides identified herein by one or more chemical
steps. Such derivatized molecules include, for
example, those molecules in which free amino groups
20   have been derivatized to form amine hydrochlorides, P-
toluene sulfoamides, benzoxycarboamides, T-butyloxycar-
boamides, thiourethane-type derivatives, trifluoroacet-
ylamides, chloroaceamides, or formamides. Free carbox-
yl groups may be derivatized to form salts, methyl and
25   ethyl esters or other types of esters or hydrazides.
Free hydroxyl groups may be derivatized to form O-acyl
or O-alkyl derivatives. The imidazole nitrogen of
histidine may be derivatized to form N-im-benzylhis-
tidine. Also included as chemical derivatives are
30   those polypeptides which contain one or more naturally
occurring amino acids derivatives of the 20 standard
amino acids. For example, 4-hydroxyproline may be
substituted for proline; 5-hydroxylysine may be
substituted for lysine; 3-methylhistidine may be
35   substituted for histidine; homoserine may be
substitut d for serin , and ornithine may be
substituted for lysine.

- 25 -

It should be understood that the present invention
is not limited to the illustrative polypeptides
depicted in Table 2 and Table 9, instead, a polypeptide
falling within the scope of this invention may extend

5   outside of, or comprise less than, the region between
amino acid 28 and amino acid 50 of coxsackie virus P2-
C, or between amino acid 250 and amino acid 273 of
GAD$_{65}$, or between amino acid 258 and amino acid 281 of
GAD$_{67}$, as well as the region between amino acid 78 and

10   amino acid 97, or between amino acid 247 and amino acid
266, or between amino acid 335 and amino acid 356, or
between amino acid 479 and amino acid 498, or between
amino acid 509 and amino acid 528, or between amino
acid 524 and amino acid 543, or between amino acid 539

15   and amino acid 556, or between amino acid 564 and amino
acid 583 of GAD$_{65}$, as long as a substantial part of a
given polypeptide is characterized by an amino acid
sequence from that region, or segments or combinations
thereof, and the polypeptide demonstrates the desired

20   immunological or biological activity against autoimmune
disease. In addition, polypeptides according to this
invention include those having amino acid sequences
which are longer or shorter in length than those of the
polypeptides illustrated in Table 2 and Table 9, or

25   which comprise segments or combinations thereof, as
long as such polypeptides consist substantially of the
region between the amino acids illustrated in Table 2
and Table 9 and demonstrate immunological or biological
activity. All polypeptides of the invention should not

30   stimulate or enhance the autoimmune disease.
Accordingly, it should be understood that the
specific selection of any one polypeptide within the
polypeptides of the invention does not involve undue
experimentation. Such a selection may be carried out.

35   by taking a number of polypeptides and testing them for
their immunological and biological activity in
ameliorating the autoimmune disease or for detecting

- 26 -

antibody. The NOD mouse represents an excellent and
w 11 characterized model for screening polypeptides of
the invention capable of ameliorating or preventing
diabetes. Example 7 illustrates an acceptable
5   procedure for routine screening of candidate
polypeptides with biologic activity.

The polypeptides according to the present invention
may be prepared by recombinant techniques or by
conventional synthesis using known polypeptide
10   synthetic methods, including synthesis on a solid
support. An example of a suitable solid phase
synthetic technique is that described by Merriweather
(*J.Am.Chem.Soc.*, 85:2149, 1963). Other polypeptide
synthetic techniques may be found, for example, in
15   Bodanszky, *et al.*, *Peptide Synthesis*, John Wiley &
Sons, 2d ed., 1976, as well as other references known
to those skilled in the art. A summary of polypeptide
synthesis techniques can be found in Stewart, *et al.*,
Solid Phase Peptide Synthesis, Pierce Chemical Company,
20   Inc., Rockford, Ill., 1984. The synthesis of
polypeptides by solution methods may also be used, for
example, as described in *The Proteins*, Vol. II, 3d ed.,
Neurath, *et al.*, eds., 105, Academic Press, New York,
NY, 1976. Appropriate protective groups for use in
25   such synthesis can be found in the above references as
well as in J. McOmie, *Protective Groups in Organic
Chemistry*, Plenum Press, New York, NY, 1973.

The polypeptides of the invention may also be
prepared in an appropriate host transformed with DNA
30   sequences that code for the desired polypeptide. For
example, a polypeptide may be prepared by the
fermentation of appropriate hosts that have been
transformed with and which express a DNA sequence
encoding the polypeptide. Alternatively, a DNA
35   sequence encoding several of the polypeptides of this
inv nti n may be link d together and those s quenc s
may then be used to transform an appropriate host to

- 27 -

permit the expression of polypeptides involved in the autoimmune disease.

The dosage ranges for the administration of the GAD polypeptides of the invention are those large enough to
5   produce the desired effect in which the symptoms or cellular destruction of the autoimmune response are ameliorated. The dosage should not be so large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like.
10   Generally, the dosage will vary with the age, condition, sex, and extent of the disease in the patient and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any counterindications.
15   Dosage can vary from about 0.1mg/m^2 to about 2000mg/m^2, preferably about 0.1mg/m^2 to about 500mg/m^2/dose, in one or more dose administrations daily, for one or several days.

The GAD polypeptides of the invention can be
20   administered parenterally by injection or by gradual perfusion over time. The GAD polypeptides of the invention can be administered intravenously, intraperitoneally, intra-muscularly, subcutaneously, intracavity, transdermally, intranasally, or enterally.
25   Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such
30   as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated
35   Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, lectrolyte replenishers (such as those bas d on Ringer's dex-

- 28 -

trose), and the like. Preservatives and other
additives may als b present such as, for exampl ,
antimicrobials, anti-oxidants, chelating agents, and
inert gases and the like.

5   The invention also relates to a method for
preparing a medicament or pharmaceutical composition
comprising the GAD$_{65}$ polypeptides of the invention, the
medicament being used for therapy of autoimmune
response to GAD$_{65}$.

10   The above disclosure generally describes the
present invention. A more complete understanding can
be obtained by reference to the following specific
examples which are provided herein for purposes of
illustration only and are not intended to limit the

15   scope of the invention.

EXAMPLE 1

CLONING AND EXPRESSION OF GAD$_{65}$

20   **A.   RECOMBINANT DNA PROCEDURES**

In order to obtain cDNA probes specific for GAD$_{65}$
and GAD$_{67}$, total RNA was extracted from adult rat brain
by guanidine isothiocyanate-cesium gradient using the
method of Chirgwin, et al. (Biochemistry, 18:5294,

25   1979). Poly (A) RNA was purified on oligo dT
cellulose, using the protocol by Bethesda Research
Laboratories (BRL). First strand synthesis was
performed by using MMLV-reverse transcriptase (BRL),
with conditions suggested, except that poly d(N$_6$)-mers

30   (Pharmacia) were used as primers. This cDNA-RNA
mixture was heat inactivated at 65°C for 15 min and
stored at -20°C. For PCR, 1/50 of the sample was added
to the 100μl reaction. Degenerate oligonucleotides
were synthesized (Applied Biosystems) to encode the

35   underlin d common amino acid sequ nces f feline (from
cDNA) (Kobayashi, t al., J.Neurosci., 7:2768, 1987)
and rat (from peptides) (Chang and Gottlieb,

- 29 -

J.Neurosci., <u>8</u>:2123, 1988) GAD (Figure 1). The 5'-end
sequence of each degenerate oligonucleotide contained
one strand of the DNA sequence recognized by either
SstI and HindIII (5' oligo) or SstI and SstII (3'-end
5   oligo). These primers were used for selective
amplification by polymerase chain reaction of the
generated cDNA template as described by Gould, *et al.*
(*Proc.Natl.Acad.Sci.,USA*, <u>86</u>:1934, 1989). PCR products
were subcloned into HindIII/SstI double digested
10   Bluescript SK vector (Stratagene), transformed into DH5
(BRL), and plated by standard methods (Maniatis, et
al., Molecular Cloning: A Laboratory Manual, Cold
Spring Harbor Laboratory, Cold Spring Harbor, NY,
1989).

15   Colony hybridization was done with an $5'-^{32}P$ end
labeled oligonucleotide specific to feline GAD_{67}
(Kobayashi, *et al.*, *J.Neurosci.*, <u>7</u>:2768, 1987). End
labeling of oligonucleotide, hybridization conditions,
and washing conditions were done as described (Wallace,
20   et *al.*, in *Guide to Molecular Cloning Techniques;*
Berger, *et al.*, Eds. in *Methods of Enzymology;* Abelson,
et al., Eds. *Academic Press, Inc.*, San Diego, 432-442,
1987), except that the nitrocellulose filters were
washed at 50°C for 15 min. Colonies which were
25   positive and negative in the hybridization were
individually picked and grown overnight in Terrific
Broth (Tartof, *et al.*, *Focus,* <u>9</u>:12, 1987). DNA was
isolated using a boiling method (Maniatis, *et al.*,
Molecular Cloning: A Laboratory Manual, Cold Spring
30   Harbor Laboratory, Cold Spring Harbor, NY, 1989) and
templates were denatured by 0.2N NaOH and purified by
Sephacryl S400 spun columns (Pharmacia). Sequencing of
denatured double stranded template was by the
chain-termination method (Sanger, *et al.*,
35   *Proc.Natl.Acad.Sci.,USA*, <u>74</u>:5463, 1977) using the
T7-sequencing kit (Pharmacia).

- 30 -

As shown in Figure 1, PCR-generated rat GAD_{65} and
GAD_{67} cDNAs were used as probes to screen a lambda ZAP
(Stratagene) rat hippocampus library provided by S.
Heinemann (Salk Institute) by standard techniques
5   (Maniatis, et al., *Molecular Cloning: A Laboratory
Manual*, Cold Spring Harbor Laboratory, Cold Spring
Harbor, NY, 1989). A 2400 nucleotide GAD_{65} cDNA (the
largest clone) was isolated and subcloned by "zapping"
as described by Stratagene. When a rat GAD_{67} cDNA was
10   obtained which was smaller than a 3.2kb rat GAD_{67} cDNA
clone already on hand, the larger cDNA was sequenced.
Exo III deletions (Henikoff, *Gene*, 28:351, 1984) were
made in both directions for GAD_{65} and GAD_{67} and templates
were prepared and sequenced as described above.
15   Anchored PCR (Frohman, et al., *Proc.Natl.Acad.Sci.,USA,*
85:8998, 1988) was done to clone the remaining 5'-ends
of GAD_{65} and GAD_{67} mRNAs which were not represented in
the original cDNA clones isolated in the library
screening. Sequencing of these clones revealed that
20   neither GAD_{65} nor GAD_{67} mRNAs contained any further
initiation codons (AUGs) in frame with the previously
designated initiation codons of the original cDNA
clones.

25   **EXAMPLE 2**

CHARACTERIZATION OF CLONED GAD_{65}

A. NORTHERN BLOT HYBRIDIZATION
Two PCR-derived cDNA probes were hybridized to
30   Northern blots containing rat brain RNA in order to
determine whether the GAD_{67} and GAD_{65} cDNAs were derived
from two different mRNAs. RNA was extracted as
described in Example 1. Poly (A) RNA was separated by
electrophoresis in formaldehyde and transferred onto
35   Biotrans (ICN) membranes, and hybridization was
performed as described by Well, t al. (*J.Neurosci.,*
16:311, 1986), xcept that 100μl/ml of poly (A) was

- 31 -

added. Probes were labeled to approximately 10^9 dpm/μg
by the oligolabeling procedure of Feinberg and
Vogelstein (Anal.Biochem., 132:6, 1983). Identical
results were subsequently obtained with full-length
5   clones of GAD_{65} and GAD_{67} cDNAs.

As shown in Figure 5, lanes 1 and 2 contain 1μg of
poly (A) selected RNA extracted from rat cerebellum.
Lane 1 was hybridized to a cDNA probe for the rat
cognate of feline GAD_{67} (Kobayashi, et al., J.Neurosci.,
10   7:2768, 1987) and lane 2 with a cDNA probe for the rat
peptide sequence (which corresponds to GAD_{65}).

The cDNA probe for the rat peptide sequence
hybridized to a 5.7kb RNA, while the cDNA probe for the
rat cognate of feline GAD_{67} cDNA, hybridized to a 3.7kb
15   RNA. This demonstrates that GAD_{65} and GAD_{67} are not
derived from the same mRNA.

B.   GENOMIC HYBRIDIZATION OF GAD_{67} AND GAD_{65}

In order to investigate the possibility that GAD_{67}
20   and GAD_{65} arise from separate genes, cDNAs of both GAD_{67}
and GAD_{65} were hybridized to DNA blots containing
genomic DNA.

For Southern blots, genomic DNA was extracted from
rat liver as described (Kaiser, et al., in DNA Cloning,
25   vol.I, A Practical Approach, D.M. Glover ed., IRL
Press, Oxford, pp. 38-40, 1985). DNA (10μg/sample) was
digested to completion with EcoRI and HindIII using
conditions recommended by the suppliers (BRL,
Gaithersburg, MD). DNA fragments were separated by
30   electrophoresis at 1.5v/cm for 16 hrs in 0.8% agarose.
The DNA was then transferred to Zeta-Probe membranes
(Bio-Rad), hybridized, and washed, as described by
Gatti, et al. (Biotechniques, 2:148, 1984), except that
5μg/ml Carnation dried milk was substituted for
35   Denhardt's solution. Probes for Southern blots w re
lab led as described in Example 1, above.

- 32 -

As shown in Figure 6, genomic DNA digested with
HindIII and EcoRI are in lanes 1 and 3 and lanes 2 and
4, respectively. GAD$_{67}$ cDNA was hybridized to lanes 1
and 2, whereas GAD$_{65}$ cDNA was hybridized to lanes 3 and
5   4. Numbers along the side of the gel are the DNA
fragment sizes in kilobases.

This data shows that the two cDNAs hybridize to
genomic fragments of different sizes. In addition, the
greatest contiguous stretch of identical nucleotide
10   sequence of GAD$_{65}$ and GAD$_{67}$ cDNAs is only 17 nucleotide
bases in length. Thus, GAD$_{67}$ and GAD$_{65}$ are encoded by
two distinct genes.

C.   ENZYMATIC COMPARISON OF GAD$_{67}$ AND GAD$_{65}$

15   Studies were done comparing the effect of PLP on
the activity of GAD$_{67}$ and GAD$_{65}$. In so doing, both cDNAs
were subcloned into vectors that allowed their
expression in bacteria (Studier, et al., J.Mol.Biol.,
189:113, 1986). Overexpression of "fusionless" GAD$_{65}$
20   and GAD$_{67}$ was accomplished by subcloning GAD$_{65}$ cDNA into
the NcoI site of pET-8c and GAD$_{67}$ cDNA into the NheI
site of pET-5c vectors (Studier, et al., J.Mol.Biol.,
189:113, 1986).

To obtain compatible sticky ends for correct in-
25   frame subcloning of both cDNAs, selective amplification
was performed by PCR using conditions suggested by
United States Biochemical (USB), with 200μM dNTPs and
1.5mM MgCl$_2$ in the mixture and annealing at 55°C with 20
cycles to decrease infidelity of AmpliTAQ (USB).
30   Primers specific for GAD$_{65}$ and GAD$_{67}$ contained one DNA
strand of the NcoI and SpeI recognition sites,
respectively. Since there is a NheI restriction site
within the coding region of GAD$_{67}$, SpeI (which is
compatible with NheI) was used.

35   PCR products were subcloned into their respective
pET v ctors, transformed into DH5 and plat d as
describ d (Maniatis, et al., Molecular Cloning: A

Laboratory Manual, Cold Spring Harbor Laboratory, Cold
Spring Harbor, NY, 1989). Colonies were picked and
grown overnight in LB broth with 50µg/ml ampicillin.
Subclones with correct orientation were transformed
into BL21(DE3) strain (Studier, *et al., J.Mol.Biol.*,
189:113, 1986) for overexpression. As a negative
control, the pET-8C vector with no insert was
transformed and subsequently induced. Single colonies
were picked, grown, induced by 1mM isopropyl-B-D-
thiogalacto-pyranoside (IPTG), and analyzed on SDS-PAGE
gels as described (Sambrook, *et al., Molecular Cloning
a Laboratory Manual*, Cold Spring Harbor Laboratory
Press, Cold Spring Harbor, 17.15-17.16, 1989).

To measure GAD activity, we induced 10ml cultures
of bacteria at OD_{600}-0.5 with 1mM IPTG. Two hours after
induction, bacteria was spun down and resuspended and
sonicated in 1ml of homogenizing buffer (1mM
phenylmethylsulfonyl fluoride (PMSF), 1mM 2-
aminoethylisothiouronium bromide (AET), and 60mM
potassium phosphate, pH 7.1). After sonication, cell
debris was removed by centrifugation and protein
concentration was measured (Bradford, *Anal.Biochem.*,
72:248, 1986) in the supernatant (supernatant was
stored in aliquots at -70°C). Brain homogenates were
prepared as described (Legay, *et al., J.Neurochem.*,
46:1478, 1986). GAD activity was measured as described
(Krieger, *et al., J.Neurochem.*, 33:299, 1984) with
0.2mM PLP present or absent and 20µl of brain
homogenate or bacterial lysate in the incubation
mixture. Production of $^{14}CO_2$ in bacterial lysates was
linear with respect to time of incubation and protein
concentration.

- 34 -

TABLE 3

Source	GAD Specific Activity[a]		Fold Increase
	- PLP	+ PLP	in Induction
BL21(DE3) + pET-8c	12 ± 0.4	9 ± 1	--
BL21(DE3) + pET-GAD$_{65}$	115 ± 3	773 ± 61	6.7
BL21(DE3) + pET-GAD$_{67}$	160 ± 2	389 ± 8	2.4
Rat Brain	131 ± 5	216 ± 2	1.6

[a] cpms of $^{14}CO_2$/μg protein/hr of
      triplicates ± S.E.M.

As shown in Table 3, bacterial lysates containing
GAD$_{65}$ or GAD$_{67}$ catalyze the conversion of $[1-^{14}C]$-
glutamate to GABA and $^{14}CO_2$.

PLP stimulates the enzymatic activity of GAD$_{65}$ more
than GAD$_{67}$. This greater stimulation probably reflects
the faster cycling of GAD$_{65}$ through the inactivation
cycle proposed by Martin and coworkers (Martin,
Cell.Mol.Neurobiol., *7*:237, 1987). This faster cycling
suggests that GAD$_{65}$ contributes more to the pool of
apo-GAD that exists *in vivo* (Miller, et al., *Brain
Res.Bull.*, *5*(Suppl.2):89, 1980). Thus, *in vivo*, PLP
appears to regulate GAD$_{65}$ activity more than GAD$_{67}$
activity.

GAD$_{65}$ activity in bacterial lysates is similar to
the five-fold PLP stimulation of GAD activity found in
synaptosomes prepared from rat substantia nigra
(Miller, et al., *J.Neurochem.*, *33*:533, 1979). Because
both GADs are more dependent upon added PLP in bacteria
than is the GAD activity in crude rat brain
homogenates, the endogenous PLP concentration of
bacteria lysates may be less than rat brain
homogenates.

- 35 -

D. IMMUNOLOGICAL IDENTIFICATION OF GAD$_{65}$ AND GAD$_{67}$

Rat brain homogenates and bacterial lysates were
extracted as described above. Equal volumes of loading
buffer were added to each sample as described (Harlow,
5   et al., Antibodies, A Laboratory Manual, Cold Spring
Harbor Laboratory, Cold Spring Harbor, NY, 1988).
Proteins were separated by electrophoresis in a 10%
acrylamide gel in SDS and electrophoretically
transferred to nitrocellulose (Harlow, et al.,
10   Antibodies, A Laboratory Manual, Cold Spring Harbor
Laboratory, Cold Spring Harbor, NY, 1988). The
unreacted sites were blocked with a phosphate buffered
saline (PBS) solution containing 2% bovine serum
albumin (fraction V), 1% gelatin, and 1% Triton-X-100
15   at 42°C for one hr. After washing, the nitrocellulose
filter was then cut into three sections and incubated
with the following primary antibodies: lanes 1 to 4
with a 1/2000 dilution of the antiserum of Oertel, et
al. (Neuroscience, 6:2689, 1981), which recognizes both
20   GAD$_{67}$ and GAD$_{65}$; lanes 5-8 with a 1/2000 dilution of K-2
antiserum, which recognizes only GAD$_{67}$; lanes 9-12 with
a 1/2000 dilution of GAD-6 monoclonal antibody, which
is specific for GAD$_{65}$ (Chang, et al., J.Neurosci.,
8:2123, 1988). All filters were extensively washed and
25   appropriate secondary antibodies were incubated and
washed. Bound antibodies were detected with ^{125}I-labeled
protein A and autoradiography. Each lane contained the
following: lanes 1, 5, and 9 are BL21(DE3) + pET-GAD$_{67}$;
lanes 2, 6, and 10 are BL21(DE3) + pET-GAD$_{65}$; lanes 3,
30   7, and 11 are rat brain homogenate; and lanes 4, 8, and
12 are BL21(DE3) + pET-8c.

The immunoblots of bacterially produced GAD$_{65}$ and
GAD$_{67}$ demonstrated that GAD$_{65}$ indeed corresponds to the
smaller GAD in brain extracts, and GAD$_{67}$ to the larger
35   form (Figure 7). Previous work has demonstrated the

- 36 -

correspondence of GAD_{67} to the larger GAD for feline
GAD_{67}, and for mouse GAD_{67} (Katarova, et al.,
Eur.J.Neurosci., 2:190, 1990; 235, 1987). The
mobilities of bacterially produced GAD_{65} and GAD_{67} (as
5   detected with the antiserum of Oertel, et al.
(Neuroscience, 6:2689, 1981) are identical to the
immunoreactive doublet seen in rat brain homogenate.

The smaller molecular weight and larger molecular
weight forms of GAD in rat brain are thus identical in
10   antigenicity and size to the products of GAD_{65} and GAD_{67}
cDNAs, respectively. Consequently, the two GADs in rat
brain are GAD_{65} and GAD_{67}. From these data it can also
be concluded that the molecular identity of the
reported PLP-dependent and PLP-independent GADs by
15   Tapia (Bayon, et al., J.Neurochem., 29:519, 1977) are
GAD_{65} and GAD_{67}, respectively. Martin and coworkers
(Spink, et al., Brain Res., 421:235, 1987) have
reported the existence of four kinetically different
forms of rat brain GAD. However, immunoblotting
20   experiments (with the antisera used here) of these
forms have not been reported.

E.   **DISTRIBUTION OF GAD_{65} and GAD_{67} IN RNAs IN BRAIN
TISSUE**
25   Experiments were done to determine the distribution
of GAD_{65} and GAD_{67} in RNAs in cerebellum using in situ
hybridization.

Transcripts of, respectively, 3.2kb and 2.3kb from
GAD_{65} and GAD_{67} cDNAs, were radiolabeled with ^{35}S
30   according to Wuenschell, et al.
(Proc.Natl.Acad.Sci.,USA, 83:6193, 1986) procedure.
Hydrolyzed fragments of 200 bp were hybridized to
coronal sections of a rat cerebellum. Animals were
anesthetized under halothane and decapitated. The
35   brain was rapidly frozen in dry ice and coronal frozen
secti ns (12μm) were fixed for 30 min in freshly
prepared 4% formaldehyde in phosphate-buffered salin

- 37 -

(PBS; 130mM NaCl, 10mM Na phosphate, pH 7.0). The
tissue was dehydrated through graded ethanol solutions
and stored at -70°C.

In order to increase tissue permeability, the
5   sections were submitted to the following pretreatments:
rehydration through graded ethanol solutions (5 min
each in 95%, 85%, 70%, 50%, and 30% ethanol); PBS (5
min); 0.02N HCl (10 min); PBS (5 min); 0.01% Triton N-
101 in PBS (1 min); PBS (2 x 5 min); 1μg/ml proteinase
10   K (7.5 min); and glycine (to inhibit proteinase K) in
PBS (3 x 5 min). Proteinase K was digested for 30 min
at 37°C before use. Sections were then incubated at
37°C in 50% formamide, 750mM NaCl, 25mM EDTA, 0.2% SDS,
0.02% BSA, 0.002% Ficoll, 0.02% polyvinylpyrrolidone,
15   250μg/ml yeast tRNA, 250μg/ml poly A, and 25mM PPES (pH
6.8).

For the hybridization, 100mM DTT, 10% dextran
sulfate, and sense or antisense ^{35}S-RNA were added to
the prehybridization solution. An aliquot (50μl) of
20   the hybridization solution containing about 3 ng (10[6]
cpm) of probe (sense or antisense) was added onto the
slides. Each slide was coverslipped and incubated for
16 hrs at 50°C, following which the siliconized
coverslips were removed by brief washing in 4 x SSC (1
25   x SSC - 150mM NaCl, 60mM Na citrate, pH 7.0).

Sections were then treated with ribonuclease A
(50μg/ml in 0.5M NaCl, 10mM Na thiosulfate, 1mM EDTA,
10mM TrisHCL, pH 8.0) for 20 min at 37°C and rinsed for
2 hrs at room temperature in 2 x SSC, 10mM Na
30   thiosulfate, for 30 min at 55°C. Sections were
dehydrated in ethanol, delipidated in xylene, coated
with Kodak NTB2 emulsion and exposed for 10 days at
4°C. The emulsion was developed with Kodak D19, and
the tissue counterstained with cresyl violet.

35   Autoradiographic grains were detected using
reflected polarized light and grain numbers, densities,
nd cell areas were det rmined with an Analytic Imaging

- 38 -

Concepts image analyzer system. Due to the low
background level, the criteria for defining a cell
"labeled" was based on the presence of more than 5
clustered grains. The GAD labeled cells were found
5   scattered throughout the brain, enabling the
measurement of the number of grains over individual
cells. The boundary of the cell and the area covered
by a grain allowed the calculation of the number of
grains per cell. This analysis was done at a high
10   magnification (800X), using both reflected polarized
light and transmitted light to simultaneously visualize
the stained cell and the superimposed grains. Numbers
are means ± S.E.M. of "n" cells.

TABLE 4

15

CELL TYPE	GRAINS/CELL		
	GAD_{67} mRNA	GAD_{65} mRNA	GAD_{67}:GAD_{65}
Purkinje	172 ± 34 (87)[a]	43 ± 2 (70)	4.0
Golgi II	96 ± 8 (80)	64 ± 9 (65)	1.5
Basket	61 ± 12 (102)	16 ± 1 (57)	3.8
Stellate	55 ± 15 (65)	18 ± 3 (37)	3.1

[a] ± S.E.M.(n)

30   In all neuronal types GAD_{67} mRNA levels are greater.
The observations with in-situ hybridization are
consistent with previous findings (Nitsch,
J.Neurochem., 34:822, 1980; Denner, et al.,
J.Neurochem., 44:957, 1985; Itoh, et al., Neurochem.
35   Res. 6:1283, 1981) that the ratio of PLP dependent to
independent GAD activities in the cerebellum is one of
the lowest in brain regions tested. In addition, as
shown in Table 3, the order of amounts for GAD_{67} mRNA is
Purkinje > Golgi II > Basket > Stellate cells; in
40   c ntrast, for GAD_{65} mRNA, this ord r is Golgi II >
Purkinje > Basket > Stellate c lls.

Th expressi n of GAD_{65} and GAD_{67} mRNAs thus differs
among classes of neurons. The contribution of each to

- 39 -

total GAD activity in turn affects how GABA production
is regulated. For example, the substantia nigra
contains one of the highest ratios of PLP-dependent to
PLP-independent GAD activities (Nitsch, *J. Neurochem.*,
5   <u>34</u>:822, 1980). Increasing GABA concentration in the
substantia nigra by local injection of inhibitors of
GABA catabolism is especially effective in reducing
seizure susceptibility (Gale, *Fed. Proc.*, <u>44</u>:2414,
1985). Experimental animals undergoing seizures
10   induced by PLP-antagonists may therefore be unable to
inhibit seizure propagation because of inhibition of
GAD_{65} particularly in nerve terminals within the
substantia nigra.

15   **F.   SUBCELLULAR LOCATION OF GAD_{65} AND GAD_{67}**

The distribution of GAD_{65} and GAD_{67} was evaluated in
the S_2 and synaptosome subcellular fractions. S_2 is a
high speed supernatant consisting of the cytosol of all
cells in the brain, while the synaptosomal fraction
20   consists primarily of nerve endings (Gray, et al., *J.
Anat., Lond*, <u>96</u>:79, 1962). For these studies, whole
rat brain fractionation was performed as described by
Booth and Clark (Booth, et al., *Biochem. J.*, <u>176</u>:365,
1978). Protein concentrations were determined by
25   Schaffner and Weissman (Schaffner, et al., *Anal.
Biochem.* <u>56</u>:502, 1973). Samples were prepared as
described (Kaiser, et al., *DNA Cloning, Vol. I, A
Practical Approach, D.M. Glover ed.* (IRL Press, Oxford,
1985, pp. 38-40), and immunoblotting was done as
30   described above using GAD-6 monoclonal antibody and K-2
antiserum. Equal amounts of protein ($16\mu g$) were added
to each lane. Autoradiography showed a linear response
of increasing amount of ^{125}I-protein A bound to antibody
with protein concentrations of 1, 3·, 10, 30, $100\mu gs$
35   with both K-2 antis rum and GAD-6 monoclonal antibody
(data not shown).

- 40 -

The results showed that GAD_{67} was present in equal
amounts in both fractions. Since the S_2 fraction
contains the cytosolic proteins of glial (as well as
other non-neuronal) and neuronal cells, the
5   concentration of GAD_{67} must be greater in neuronal cell
bodies than in nerve endings. In contrast, the
concentration of GAD_{65} was greater in synaptosomes than
in S_2. These subcellular fractionation experiments
suggest that, in contrast to GAD_{65}, a much greater
10   fraction of GAD_{67} is present in cell bodies of neurons
than in nerve terminals. Thus, subcellular
fractionation, like immunohistochemistry, shows that
GAD_{65} and GAD_{67} have different subcellular distributions.

In vivo experiments utilizing inhibitors of GABA
15   synthesis and degradation have suggested that the GABA
pool in neuronal cell bodies is different from that in
the nerve terminals (Iadarola, et al., *Mol. Cell.*
Biochem., 39:305, 1981). GABA produced by GAD_{67} may be
involved more in cellular metabolism (for example, in
20   the GABA shunt) and in dendrodendritic synapses. The
dendrites of granule cells in the olfactory bulb; which
form dendrodendritic synapses with mitral dendrites
(Shepard, *Physiol. Rev.*, 52:864, 1972) and probably
release GABA (McLennan, *Brain Res.*, 29:177-184, 1971),
25   label intensely with K-2 antiserum. While not shown
here, it has also been found greater GAD_{67} than GAD_{65}
mRNA levels (2-3 fold) in the olfactory bulb. This
distribution is consistent with the reported finding
that most GAD activity in the olfactory bulb is present
30   in S_2 and P_1 (crude nuclear pellet) and not in
synaptosomes (Quinn, et al., *J. Neurochem.*, 35:583,
1980).

The differing subcellular distributions of GAD_{65} and
GAD_{67} could result from cytoskeletal anchoring or from
35   some unknown protein targeting mechanism. Som
cytoskeletal proteins have distributions that resemble
GAD_{65} and GAD_{67}. For exampl , in cultured sympathetic

- 41 -

neurons Peng, *et al.* (*J Cell. Biol.*, <u>102</u>:252, 1986),
demonstrate that 84% of tau is in axons while 100% of
MAP-2 is in cell bodies and dendrites. In addition,
43kd protein, a cytoskeletal protein, is thought to
5   anchor the acetylcholine receptor to the underlying
membrane cytoskeleton (Flucher, *et al.*, *Neuron*, 3:163,
1989).

EXAMPLE 3

10   **DETECTION OF GAD AUTOANTIBODIES IN CLINICAL SPECIMENS**

A. MATERIALS AND METHODS

1. Patient Specimens. Sera from four groups of
individuals were selected from a previous study by
Atkinson and co-workers (Atkinson, *et al.*, *Lancet*,
15   <u>335</u>:1357-1360, 1990). These groups consisted of:
Group (1), 1 new onset IDD patients diagnosed according
to the established National Diabetes Data Group (NDDG)
criteria (Gleichman, *et al.*, *Diabetes*, <u>36</u>:578-584,
1987) that had been referred to the University of
20   Florida, Diabetes Clinics; Group (2), 5 randomly
selected islet cell cytoplasmic antibody (ICA) negative
non-diabetic controls without any known family history
of autoimmune disease; Group (3), 13 individuals whose
sera had been collected 3 to 66 months prior to their
25   documented clinical onsets of IDD; Group (4), non-
diabetic controls and relatives, and those who were
studied prior to their onsets of IDD; and Group (5), 3
patients at risk for IDDM, but where onset has not yet
occurred. This latter group had been ascertained
30   through ongoing prospective ICA screening studies of
more than 5000 first degree relative of IDD probands,
and 8200 individuals from the general population (of
which 4813 were school children).

- 42 -

2. **Islet Cell Aut antibodi s.** ICA were assayed by indirect immunofluorescence on blood group O cryocut pancreatic (Atkinson, *et al., Lancet*, 335:1357-1360, 1990). All results were interpreted on coded samples,
5   with control negative and positive sera in each batch. The degrees of ICA positivity were analyzed with the guidelines established by the Immunology Diabetes Workshop (IDW) *for* the standardization of ICA (Gleichman, *et al., Diabetes*, 36:578-584, 1987). All
10   positive sera were titered by end point dilution, and the Juvenile Diabetes Foundation (JDF) units were determined by reference to a standard serum previously calibrated to the international JDF standard of 80 units. In the studies reported here, a positive ICA
15   result was defined by replicate titers of 10 JDF units or greater.

3. **HLA DR Typing.** HLA DR typing was performed as adapted from the method described by Van Rood and Van Leuwen (*Nature*, 262:795-797, 1976), using DR trays (One
20   Lamda Laboratories, Los Angeles, CA).

4. **Human Islet Cells.** Human pancreatic islets were isolated from cadaveric pancreases and maintained *in vitro* as previously described (Ricordi, *et al.,*
25   *Diabetes*, 37:413-420, 1988). The islet cells were metabolically labeled with ^{35}S methionine (Amersham, Arlington Heights, IL) *in vitro* (95% air/5%CO_2).

5. **Islet Cell Extractions and Immunoprecipitations.** Islet cells were extracted as previously described by
30   Atkinson, *et al.* (*Lancet*, 335:1357-1360, 1990) with the following modifications. For immunoprecipitation studies, the islet cell lysates were precleared twice by incubation (2h, 4°C) with either control, IDD serum (100μl), or GAD-6 (Chang, *et al., J.Neuro*, 8:2123-2130,
35   1988) (1μl in 99μl of Tris buffer (Atkinson, *et al., Lancet*, 335:1357-1360, 1990) for every 1000 islets. Immun compl xes were then absorbed (1h 4°C) with an

- 43 -

excess f protein A Sepharose CL-4B (Pharmacia, NJ).
Aliquot volumes representing 1000 islet cells
containing unbound (precleared) lysate were then
incubated (12h, 4°C) with either IDD or control sera
5   (25μl), or GAD-6 (Chang, et al., J.Neuro, 8:2123-2130,
1988) (1μl in 25μl Tris buffer). Following another
incubation with protein A Sepharose CL-4B (1h, 4°C),
the complexes were then washed 5 times with 50mM Tris
HCL (pH 7.4) with 0.1% SDS, 1.0% Triton X-114, and 2mM
10   EDTA, and then washed again one time in double
distilled water. The protein A Sepharose CL-4B was
then boiled in Laemmli sample buffer (Laemmli, Nature,
227:680-685, 1970), and the samples were subjected to
SDS-PAGE and fluororadiography (Kodak, X-omat AR5)
15   using Enhance (New England Nuclear). Alternatively,
the autoradiographs were analyzed by a BETAGEN (Boston,
MA) analyzer. Both 64KA positive and negative sera
were used in each assay, to serve as interassay
controls. All fluororadiographs were analyzed and
20   rated as positive or negative after comparison with the
known interassay controls. Positive serum samples were
designated as 1 when a sample resulted in
immunoprecipitation of a low intensity 64,000 M_r band, 2
if a moderate intensity band was observed and 3 if the
25   intensity of the immunoprecipitated protein was high.
A similar rating procedure was employed for the
intensity of bands corresponding to immunoprecipitated
^{35}S-GAD$_{65}$ and ^{35}S-GAD$_{67}$.

6.   Immunoprecipitations. Immunoprecipitation of
30   bacterial lysates containing ^{35}S-GAD$_{65}$ or ^{35}S-GAD$_{67}$, and
GAD from human brain homogenate, was completed as
described above in immunoprecipitation studies of human
islet cell extractions.

7.   GAD Assays. Human brain homogenates were incubated
35   with patient sera as d scribed above in human islet
cells. Aft r absorpti n and washes, the protein A
agarose slurry was aliquoted into thr e equal volumes

- 44 -

and GAD activity was measured as described (Krieger, et al., Neurochem. 33:299, 1984). Briefly, Protein A agarose beads were incubated with ($1-^{14}C$)-glutamate (Amersham) in a designated incubation mixture (Krieger,

5   et al., J. Neurochem. 33:299, 1984) and production of $^{14}CO_2$ was quantitated by a liquid scintillation counter.

8.   **Production of ^{35}S-GAD$_{65}$ and ^{35}S-GAD$_{67}$.** Rat GAD$_{65}$ and GAD$_{67}$ cDNAs were subcloned into a bacterial expression system as previously described. Labeling of ^{35}S-GADs

10   was completed by pulsing IPTG induced bacterium (growing in Minimal Media) for 15 minutes with TRAN ^{35}S-label (ICN). Cultures were then spun down and resuspended and sonicated in 1ml of homogenizing buffer (1mM phenylmethylsulfonyl fluoride (PMSF), 1mM 2-

15   aminoethylisothiouronium Bromide (AET) and 60mM potassium phosphate, pH 7.1). After sonication, cell debris was removed by centrifugation and protein concentration was measured (Bradford, Anal.Biochem., 72:248, 1986) in the supernatant (supernatant was

20   stored in aliquots at -70°C).

B.   **IMMUNOREACTIVITY OF IDDM SPECIMENS**

       Sera from patients with IDDM were tested for the ability to precipitate GAD from human brain

25   homogenates.

TABLE 5

SERA FROM IDDM PATIENTS IMMUNOPRECIPITATE GAD ACTIVITY

Patient	IDDM	Pre-IDDM Period[1]	64K[2]	JDF[3]	GAD Activity[4] cpm's
DA	+[5]	>24	3	164	13,762
DC	+	>1	3	20	1,719
RS	+	5	3	40	588
NL	+	0	2	80	440
DM	+	>1	2	10	184
C	-	na	0	0	280
C	-	na	0	0	285
C	-	na	0	0	325
C	-	na	0	0	275
C	-	na	0	0	270

[1] Expressed as months
[2] 64K titers as described in Experimental Methods
[3] The islet cell antibody test as expressed in Juvenile Diabetes Foundation (JDF) units
[4] Not adjusted for background
[5] At risk for diabetes (also, failed glucose test)
na - Not applicable

- 46 -

As shown in Table 5, the sera of four (out of five) at risk for IDDM or IDDM patients bound significantly greater amounts of enzymatically active GAD of human brain extracts than sera from control patients. In

5   addition, sera from one of the patients was drawn in a pre-IDDM period, thus autoantibodies to GAD are present prior to the onset of IDDM symptoms (see C below).

Further experiments (results not presented) showed that the sera of two at risk IDDM patients (DA, DC)

10   immunoprecipitated recombinantly produced ^{35}S-GAD$_{65}$ whereas recombinantly produced ^{35}S-GAD$_{67}$ was only recognized by sera of patient DA (and to a lesser degree than ^{35}S-GAD$_{65}$).

Additional studies using patient DA sera showed the

15   presence of antibodies which recognize specific polypeptides produced in human pancreatic islet cells. Electrophoretic analysis of the bound polypeptides demonstrated the presence of autoantibodies to a 64kD component, as previously shown by others in human IDDM

20   (Baekkeskov, et al., Nature, 298:167-169, 1982) and in animal models (Baekkeskov, et al., Science, 224:1348-1350, 1984; Atkinson, et al., Diabetes, 37:1587-1590, 1988). Prior absorption of these sera with GAD-6 monoclonal, which recognized GAD$_{65}$ but not GAD$_{67}$, or with

25   bacterially produced GAD$_{65}$, abolished the ability of the sera to recognize the 64kD pancreatic polypeptide. The epitopes recognized by autoantibodies to the 64kD autoantigen are thus present in GAD$_{65}$, indicating that the 64kD autoantigen is indeed GAD$_{65}$. In order to

30   investigate the predictive value of GAD$_{65}$, sera drawn from patients prior to onset of clinical manifestation of IDDM were tested for autoantibodies to GAD$_{65}$.

TABLE 6

IDDM PATIENTS ANALYZED FOR AUTOANTIBODIES PRIOR TO THE ONSET OF DISEASE

Patient	Sex	HLA	Age Onset[1]	Pre-IDD Period[2]	JDF	64KD[3]	GAD[3]$_{65}$	GAD[3]$_{67}$
TA	M	3,2	17	11	20	2	0	1
CA	F	4,5	38	4	0	1	1	0
RA	M	2,1	5	34	0	2	1	0
TB	M	2,4	11	66	40	1	1	0
AB	M	N.D.	23	6	160	3	3	2
VC	F	4,6	15	3	40	1	0	1
JD	M	6,1	34	25	10	3	1	1
DR	F	3,4	14	42	320	2	1	0
JG	M	3,3	12	8	40	1	0	0
BR	M	3,3	5	9	0	0	1	1
KR	F	4,X	34	14	10	3	2	0
JT	F	4,6	7	10	N.D.	1	1	1

[1] Age of IDDM onset expressed as months

[2] The time interval between sera drawn and IDDM onset expressed as months

[3] 1 = lowest; 2 = medium; and 3 = highest band intensities

N.D. - not determined

- 48 -

As shown in Table 6, 9 out of 12 specimens (75%)
were immunoreactive with ^{35}S-GAD$_{65}$. In addition, two
patients (JA and VC) were immunoreactive to GAD$_{67}$, but
not GAD$_{65}$ under these conditions. Therefore, in
5   combination, autoantibodies to GAD$_{65}$ and GAD$_{67}$ were
present in 11 out of 12 (91%) of these patients sera.
This finding suggests that although autoantibodies to
GAD$_{65}$ are more common than autoantibodies to GAD$_{67}$, the
use of both recombinant GADs (GAD$_{65}$ and GAD$_{67}$) in an
10   assay would allow for greater predictability of IDDM.
Previous tests of these sera (Atkinson, et al., Langet,
335:1357-1360, 1990) demonstrated that 11 out of 12, or
92%, immunoreacted with the ^{35}S-64kD molecule from human
pancreatic islet cells. The serum which contained
15   detectable autoantibodies to the 64kD molecule and not
GAD$_{65}$ was a serum which contained the lowest titer
(or "1") for the 64kD molecule. Thus, the false
negative obtained was due to a lack of sensitivity in
this assay. Furthermore, this assay predicted IDDM in
20   one patient (BR) who was negative for 64K.

These results show that the 64kD molecule
identified in β-cells of human pancreas is identical in
size and antigenicity to rat GAD$_{65}$. Furthermore, sera
drawn from patients prior to IDDM onset contain
25   autoantibodies to GAD$_{65}$. Consequently, the GAD$_{65}$
recombinant molecule is of great utility as a
diagnostic tool for predicting IDDM. The ability of a
physician to diagnose IDDM prior to actual symptoms may
result in a greater extension of time before insulin
30   therapy is needed. The sensitivity of such
immunoassays will improve with the use of a recombinant
GAD$_{65}$ of human origin which represents the GAD form
present in β-cells of the pancreas.

35

- 49 -

EXAMPLE 4

IMMUNE PROLIFERATIVE RESPONSE TO POLYPEPTIDE

Polypeptides were synthesized using an automatic instrument (Applied Biosystems) and standard

5   conditions. These polypeptides were then tested to compare their relative ability to stimulate proliferation of splenic lymphocytes and islet infiltrating T lymphocytes (IITLs). In this study, polypeptides derived from the GAD_{65} core sequence and

10   from the homologous region of polio virus were compared. Appropriate cells were cultured for 5 days with the respective polypeptide in the presence of 5 X 10^4 irradiated spleen cells. ^{3}H-thymidine was added during the last 16 hours of culture.

15

-50-

TABLE 7

ANTIGEN	AMINO ACID SEQUENCE	^{3}H-THYMIDINE INCORPORATION (cpm) BY LYMPHOID CELL POPULATION	
		IITLs[a]	SPLEEN[b]
None	---	1,100	6,500
Poliovirus	MKSMCPQAQLKVKYL	900	22,500
GAD$_{65}$	ARFKMFPEVKEKGMAA	9,500	23,300

[a] islet infiltrating T lymphocytes (3 X 10^4 cells/well)

[b] 1 X 10^5 cells/well

- 51 -

In these studies, th re was no significant
differ nce in the proliferative activity of cultur s of
spleen lymphocytes exposed to either the polio or the
GAD$_{65}$ polypeptides. However, both polypeptides
5   stimulated a T cell response which was higher than that
found in the media control. The lack of difference in
proliferation in the spleen cell population may be due
to a lower frequency of GAD polypeptide specific T
cells.
10   The IITL population, when evaluated in the same
manner, showed a marked difference in cell
proliferation. In this system, the response to the
GAD$_{65}$ polypeptide was 9-fold greater than that of either
the culture media or the polio polypeptide. This data
15   strongly suggests that the GAD$_{65}$ is an important antigen
for T cell responses in the IITL population. This data
suggests that molecular mimicry plays a role in the
pathogenesis of diabetes.

20   **EXAMPLE 5**
        GAD INDUCES PROLIFERATION OF SPLEEN CELLS OF NOD MICE
                Proliferative T-cell responses to β-cell antigens
        (βCA) develop spontaneously in the nonobese diabetic
        (NOD) mouse model in a defined chronological order.
25   The NOD mouse experimental model is considered the most
        analogous *in vivo* system available for studying IDDM in
        humans. This example describes studies on the antigen-
        induced blastogenesis of spleen cells from newborn to 5
        month old female NOD mice when exposed to GAD and other
30   peptides.
                The βCAs tested included one of the two forms of
        GAD (Kaufman, et al., *Science*, 232:1138-1140, 1986;
        Erlander, et al., *Neuron*, 7:91-100, 1991; Kaufman, et
        al., *Trends in Pharm. Sci.* (in press)), (GAD$_{65}$,
35   previously kn wn as th 64K autoantigen (Baekkeskov, et
        al., *Nature*, 298: 167-169, 1981; Baekkeskov, et al.,
        Nature, 347:151-156, 1990), carboxypeptidase H (CPH)

- 52 -

(Castano, *et al., J. Clin. Endoctrinol. Metab.,*
73:1197-1201, 1991), insulin (Palmer, Predicting IDDM,
Diabetes Reviews, 1:104-115, 1993) and a peptide of hsp
which has been shown to be the immunodominant

5   determinant recognized by NOD T-cells (Elias, *Proc.
Natl. Acad. Sci.,* 88:3088-3091, 1991). GAD in
particular, is a good candidate for the initial target
antigen in IDDM since autoantibodies to GAD arise early
in the natural history of the disease (Baekkeskov,

10   *supra;* Atkinson, *et al., Lancet,* 335:1357-1360, 1990;
Kaufman, *et al., J. Clin. Invest.,* 89:283-292,1992).
Furthermore, unlike the ubiquitous hsp, GAD is
expressed primarily in β-cells and the immunologically
privileged central nervous system (CNS) and gonads. As

15   control antigens, irrelevant prototype foreign and self
antigens including hen eggwhite lysozyme (HEL), human
serum albumin (HSA), *E. coli.* β-galactosidase (β-gal)
and murine myelin basic protein (MBP) were used.

NOD (Taconic farms) and BALB/c mice (Jackson

20   Laboratories) were kept under specific pathogen free
conditions. The mice were sacrificed at the ages
indicated and the spleen cells were tested directly *ex
vivo* for their proliferative recall response to
antigen. Single cell suspensions of spleen cells were

25   plated at 1 x 10^6 cells per well in 96 well microtiter
plates in 200 μl serum free HL-1 medium (Ventrex) that
was supplemented with 2mM glutamine with or without 10
μg/ml antigen (or 7 μM peptide) in triplicate cultures.
During the last 16h of the 72h culture period, 1

30   μCi[^{3}H]-thymidine was added per well. Incorporation of
label was measured by liquid scintillation counting.

Both human GAD_{65} (Bu, *et al., Proc. Natl. Acad.
Sci.,* 89:2115-2119, 1992) and *E. coli* β-gal (control)
were purified from recombinant bacteria on the basis of

35   a hexahistidine tag which allows their rapid affinity
purification by m tal affinity chromatography (Hochuli,
et al., Bio/Technology, 6:1321-1325, 1988). Bovine CPH

- 53 -

was the generous gift of L. Fricker (Albert Einstein
Col. Med.) and human insulin was purchased from Eli
Lilly.

    As illustrated in Figure 8, while proliferative T-
5   cell responses were not detected at any time point to
the control antigens, a response to GAD arose at 4
weeks of age in NOD mice, concurrent with the onset of
insulitis in the colony. The blastogenesis induced by
GAD increased during the next four weeks and then
10 declined to background levels by week 16. At 6 weeks
of age, near the peak of anti-GAD reactivity, T-cell
responses to hsp appeared and increased until week 15
and then diminished as well (Figure 8). In all NOD
mice tested, hsp reactivity was preceded by an anti-GAD
15 response, suggesting that the former reactivity
developed as a secondary event during the autoimmune
process. Similarly, while no response was detected to
CPH at 4 weeks of age, a strong anti-CPH response was
observed by week 8. In some mice, a weak response to
20 insulin was observed at 12 weeks, which became more
prevalent at 15 weeks of age (Figure 8 and Table 8).
None of the antigens induced proliferation in T-cells
from age-matched control BALB/c or (NOD x BALB/c) F_1
mice, both of which do not develop insulitis or IDDM.
25 T-cell reactivity subsequently arises to other βCAs,
consistent with the inter-molecular diversification of
the autoimmune response. Thus, the autoimmune response
to GAD was the first to occur among the autoantigens
tested. In view of this, tolerization to GAD should
30 prevent the spread of autoimmunity to other βCAs and
insulitis. If this were not the case then tolerization
to GAD should have no effect on the response to these
other antigens.

    Blastogenesis provides an approximation of the
35 r lative clonal sizes of antigen-specific CD4+ T-cells
(Corradin, et al., J. Immunol., 119:1048-1053, 1977).
Th data in Figure 8 shows that GAD reactive T-c lls

- 54 -

"spontaneously" undergo clonal expansion concurrent with the onset of insulitis. These findings are consistent with an endogenous priming event.

EXAMPLE 6

INDUCTION OF TOLERANCE WITH GAD

This example describes a study which shows that induced tolerance to GAD can ameliorate IDDM.

1.   In these experiments female NOD mice were intravenously injected at 3 weeks of age with 50 μg GAD, β-galactosidase, mycobacterial hsp65 (m-hsp) or 0.1 μg of the immunodominant hsp peptide (hsp-p), in PBS. At 12 weeks of age, mice were examined for insulitis and autoantigen reactive T-cells. At this age both indications are established in untreated NOD mice. Pancreatic tissue sections were stained by immunoperoxidase techniques for insulin and were counterstained with hematoxylin. Insulitis was scored in a blinded manner by examining 54 to 87 islets on 5 interrupted tissue sections from each pancreas. Proliferative splenic T-cell responses induced by various antigens were performed as described above in Example 4. Data in Table 8 are expressed as the average [^{3}H]-thymidine label (cpm) incorporated in triplicate cultures.

TABLE 8A

GAD Induced Tolerance

Treatment	Insulitis Score[a]	N	Spleen Cell Proliferation (SI ± SEM)[b]							
			β-Gal	GAD	#17	#34	#35	hsp Peptide	CPH
					GAD Peptides				
Uninjected	2.4±0.2	5	1.0±0.2	9.5±2.1	4.8±0.4	6.0±0.1	2.9±0.2	8.7±1.0	ND[c]
β-Gal.	2.6±0.6	5	1.1±0.1	15.4±1.8	5.1±0.8	5.1±0.6	4.0±0.2	8.6±0.5	11.5±0.9
GAD 1.1±0.02	0.1±0.1	8	1.1±0.03	1.6±0.3	1.0±0.05	1.2±0.1	1.0±0.1	1.2±0.1	1.2±0.1
hsp-p	1.7±0.4	5	1.1±0.05	5.8±0.2	4.5±0.1	4.1±0.3	4.2±0.1	1.1±0.04	4.4±0.2
m-hsp	1.8±0.5	6	1.0±0.1	4.2±0.1	3.9±0.1	3.9±0.1	3.4±0.2	1.0±0.03	4.3±0.2

[a]   Severity of mononuclear cell infiltration was defined histologically (0 = no lymphocytic infiltration; 1 = <25%; 2 = 25-50%; 3 = 50-75%; 4 = >75%) (Qin, et al., Immunol., 150:2072-2080, 1993). Score is mean ± SE.

[b]   Significant responses noted by solid underline, borderline responses noted by double underline.

[c]   Not determined.

TABLE 8B

GAD Induced Tolerance

Treatment	Insulitis Score[a]	N	Spleen Cell Proliferation (SI±SEM)[b]		GAD Peptides				hsp Peptide	CPH
			β-Gel	GAD	#17	#34	#35			
11 peptide	≈2.5		1.0±0.1	21.1±2.2	13.7±1.5	11.4±1.5	11.3±0.7		13.3±0.9	ND
34/35 peptides (+IFA)	0.7±0.4		1.0±0.2	1.9±1.1	2.2±1.2	1.1±0.3	1.0±0.1		1.8±1.1	ND
IFA alone (+IFA)	≈2.5		1.0±0.1	8.1±0.5	5.0±0.5	4.8±0.5	4.8±0.4		6.6±0.6	ND

• Severity of mononuclear cell infiltration was defined histologically (0 = no lymphocytic infiltration; 1 =<25%; 2 = 25-50%; 3 = 50-75%; 4 =>75%) (Qin, et al., Immunol., 150:2072-2080, 1993). Score is mean ± SE.

b Significant responses noted by solid underline, borderline responses noted by double underline.

c Not determined.

TABLE 8C

GAD Induced Tolerance

Treatment	Insulitis Score[a]	N	Spleen Cell Proliferation (SI ± SEM)[b]						
			β-Gal	GAD	#17	#34	#35	hsp Peptide	CPH
					GAD Peptides				
11 peptide	≈(?)		1.4±0.4	12.1±3.5	8.9±0.4	8.4±1.0	6.8±0.3	8.7±0.9	ND
HEL peptide	(?)		1.2±0.2	10.7±3.5	8.4±1.4	5.9±1.6	6.9±1.9	8.0±1.8	ND
34/35 peptides (+IFA)	(?)		1.0±0.1	4.4±2.6	1.8±1.1	1.0±0.1	1.1±0.2	2.7±1.3	ND

a   Severity of mononuclear cell infiltration was defined histologically (0 = no lymphocytic infiltration; 1 =(25%; 2 = 25-50%; 3 = 50-75%; 4 =)75%) (Qin, et al., Immunol., 150:2072-2080, 1993). Score is mean ± SE.

b   Significant responses noted by solid underline, borderline responses noted by double underline.

c   Not determined.

Seventy five perc nt of the GAD treated mice, but none
of the controls, showed no T-cell reactivity to GAD
(indicating complete tolerization) or to other βCAs.
These mice were also completely free of insulitis
5   (score 0.0). If there were another effector T cell
population in the islets, specific for an unknown βCA,
that preceded the anti-GAD response, the release of
cytokines by this population should have promoted T-
cell responses to βCAs and insulitis (Sarvetnick, et
10   al., Nature, 346:844, 1990; Heath, et al., Nature,
359:547, 1992). Twenty five percent of the GAD-treated
mice were not completely tolerized to GAD, as evidenced
by a weak residual GAD reactivity (SI of about 3) and
displayed very limited peri-insulitis. In contrast,
15   while tolerization to both of the hsp antigens was
complete, these treatments reduced, but did not
prevent, the development of T cell responses to other
βCAs or insulitis. Thus, while the inactivation of
GAD-reactive T cells prevented β cell autoimmunity, hsp
20   tolerization only partially reduced it, as would be
expected if a secondary element was removed from the
amplifactory cascade.

In ongoing experiments examining the effects of GAD
tolerization on diabetes incidence, all of the GAD
25   treated mice (n=17, presently 37 weeks old) have normal
glucose levels, while 70% of the mice receiving control
antigens developed hyperglycemia by 19 weeks of age
(n=20). Five GAD treated mice were sacrificed at 30
weeks of age. All were free of detectable βCA reactive
30   T cells. Of these five animals, four mice were
completely free of insulitis and one mouse displayed
very limited peri-insulitis. These data show that
inactivation of GAD reactive T-cells prevents the long
term development of insulitis and diabetes.
35   2.   In a second set of exp riments neonatal female
NOD mice were injected intraperitoneally IFA with
peptid 11, a mixtur of peptides 34 and 35 plus IFA,

- 59 -

or with IFA alone and at 12 weeks of age the mice were
examined for insulitis and autoantigen reactive T-cells
as in Example 6.1. Proliferative splenic T-cell
responses induced by the various antigens were
5   performed as in Example 4, and data in Table 8B are
expressed as the average [^{3}H]-thymidine label (cpm)
incorporated in triplicate cultures.

The data in Table 8B show that tolerization with
control peptide 11 did not prevent auto antibody
10   response to GAD or to GAD peptides 17, 34 or 35. Nor
was response to hsp peptide prevented by tolerization
with peptide 11. IFA alone was somewhat effective at
suppressing immune response. By contrast, tolerization
with the mixture of peptides 34 and 35 suppressed the
15   autoimmune response of spleen cell proliferation to all
species tested: β-gal, GAD, GAD peptides 17, 34 and
35, and hsp peptide. In addition, tolerization to GAD
peptides 34 and 35 greatly reduces insulitis but does
not completely prevent it as whole GAD65 does.
20   3.   In a third set of experiments female NOD mice
were intravenously injected at three weeks of age with
peptide 11 (control), a mixture of peptides 34 and 35
plus IFA, or with HEL peptides and at 12 weeks of age
the mice were examined for insulitis and autoantigen
25   reactive T-cells as in Example 6.1. Proliferative
splenic T-cell responses induced by the various
antigens were performed as in Example 4, and data in
Table 8C are expressed as the average [^{3}H]-thymidine
label (cpm) incorporated in triplicate cultures.
30   The data in Table 8C show that tolerization with
control peptide 11 did not prevent auto antibody
response to GAD, to GAD peptides 17, 34 or 35 or to hsp
although the response was not as great as in Example
6.2. Nor was response to hsp peptide prevented by
35   immunization with peptid 11. By contrast,
immunization with th mixture of p ptides 34 and 35
plus IFA suppressed the autoimmune response of spleen

- 60 -

cell proliferation to all species test d: β-gal, GAD, GAD peptides 17, 34 and 35, and hsp peptide.

EXAMPLE 7

CHARACTERIZATION OF GAD-REACTIVE T-CELLS

5   This example describes studies on GAD-Reactive T-Cells for additional properties that distinguish activated/memory from resting/naive lymphocytes.

In one series of experiments γ interferon (IFNγ) was measured by ELISA in culture supernatants (CSN) of

10   spleen cells of 6-9 week old mice after challenge with GAD or control antigens HEL and MBP. Additionally, the frequency of antigen specific, IFNγ-producing cells was determined by an ELISA spot technique (T. Taguchi, et al., J. Immunol., 145:68-77, 1990). Frequency of

15   antigen-induced, spot forming cells (SFC) among 10^3 spleen cells is represented in Figure 9(a). Values are the mean + SEM from 5 individual female NOD mice, each tested in triplicate cultures with or without antigen. Results from a single experiment are shown. These are

20   representative of 3 separate experiments.

In performing these experiments, freshly isolated spleen cells were cultured with or without antigen as described in Example 4. CSN were taken after 48 h and the concentration of IFNγ was determined by ELISA

25   (Macy, et al., FASEB J., 3003-3009, 1988). IFNγ specific monoclonal antibody (mAb) R4-6A2 (Pharmingen) was used as the capturing reagent and biotinylated mAb XMG 1.2 (Pharmingen, also specific for IFNγ) was used in conjunction with streptavidin-alkaline phosphatase

30   (Zymed) and p-nitrophenol for detection of bound lymphokine. Recombinant murine IFNγ (Pharmingen) was used as a standard. ELISA spot assays for the detection of antigen-specific, IFNγ-producing cells were performed as described (Taguchi, et al., J.

35   Immunol., 145:68-77, 1990). After a 24h pre-activation cultur of spleen cells with our without antigen, cells w re transferred by serial dilution to 96 well

- 61 -

microtiter plates (Millipore) that had be n pre-coated
with mAb R4-6A2. After 24h, the cells w re removed and
IFNγ spots were visualized using XMG 1.2-biotin in
conjunction with nitroblue terazolium-

5   bromochloroindolyl phosphate substrate (Sigma). Spots
were counted visually and the frequency of antigen
specific cells was determined from the difference
between the number of spots seen with and without
antigen.

10   As shown in Figure 9(a), when freshly isolated T-
cells from 6-9 week old NOD mice were challenged with
GAD or control antigens, high concentrations of IFNγ
were detected only in cultures containing GAD,
suggesting that the GAD specific T-cells had been pre-

15   activated in vivo, since only pre-activated T-cells
(Th1) produce IFNγ within 48 hours after antigen
recognition (Ehlers, et al., J. Exp. Med., 173:25-36
1991; Croft, et al., J. Exp. Med., 176:1431-1437,
1992). In contrast, T-cells from age matched BALB/c

20   mice did not respond to GAD or to control antigens by
IFNγ production (data not shown).

Results of the ELISA spot assay to measure directly
the frequency of GAD-specific T-cells showed that while
in 6-9 week old NOD mice, T-cells reactive to control

25   antigens constituted approximately 1 in 10^5 cells in the
spleen, the frequency of GAD-reactive T-cells was about
two orders of magnitude higher, ranging from 90-291
cells per 10^5 cells (Figure 9(a), confirming the data
obtained by proliferation assays (Figure 8) that these

30   cells had been clonally expanded in vivo.

In another series of experiments, GAD specific T-
cells were characterized for expression of the cell
surface marker L-selectin, since murine T-cells convert
from an L-selectin+ (L-sel+) to an L-selectin⁻ (L-sel⁻)

35   phenotype upon activation (Bradley, et al., J.
Immunol., 148:324-331, 1992).

- 62 -

To perform these studies, pooled spleen cells from 3 to 4 age matched mice were panned on plates coated with goat-anti-mouse Ig (Zymed) to remove adherent macrophages as well as B cells. Next, CD8+ cells were
5   coated with mAb 58.6-72 (ATCC) and removed by panning over plates coated with goat-anti-rat Ig (Zymed). The non-adherent CD4+ cell fraction was labeled with anti-L-selectin mAb MEL-14 (ATCC) and panned on goat-anti-rat Ig coated plates. Both the adherent (CD4+ L-sel$^+$)
10   and non-adherent (CD4+,L-sel$^-$) fractions were sampled. Purity of the cell fraction was assessed by FACS analysis; cells were $>$90% CD4+ and $>$95% enriched for the L-sel$^-$ or L-sel$^+$ phenotype. The purified cell fractions were tested for GAD reactivity by seeding
15   them at 2 x 10^5 cells per well in 96 well microtiter plates with or without antigen. Irradiated (3000 rad), unseparated spleen cells of 3 week old NOD mice were added at 5 x 10^5 cells per well as a source of antigen presenting cells. Supernatants of triplicate cultures
20   were taken 48h later and their IFNγ content was determined by ELISA.

The results of this study showed that by 2-3 weeks of age, GAD reactive T-cells could not be detected in either the L-sel$^+$ or the L-sel$^-$ population, consistent
25   with a low frequency of antigen reactive precursors at this time point. However, by 6 weeks of age high levels of IFNγ were induced by GAD (but not by control antigens) in the L-sel$^-$ (but not the L-sel$^+$) subpopulation of CD4+ cells (Figure 9(b)).
30   The increase in clonal size of GAD reactive T-cells, their production of IFNγ and their L-sel$^-$ phenotype provide three independent lines of evidence that a potentially pathogenic (Ando, et al. *Cell Immunol.*, 124:132-143, 1989) Th1 type T-cell response
35   is sp ntaneously primed to GAD *in vivo* early in NOD development.

- 63 -

EXAMPLE 8

CHARACTERIZATION OF GAD SPECIFIC T-CELL
DETERMINANT RECOGNITION

The fine specificity of the anti-GAD T-cell

5   response was mapped using a set of 38 peptides
(numbered successively from the N-terminus) that were
20-23 amino acids (aa) long and span the entire GAD_{65}
(Bu, et al., Proc. Natl. Acad. Sci., 89:2115-2119,
1992) sequence with 5aa overlaps (Figure 10).

10   Spleen cells were tested from 4 (Figure 10a), 5
(Figure 10b) and 7 (Figure 10c) week old NOD mice for
proliferative responses (as described in Example 4) to
the GAD peptides. Peptides were present in cultures at
7 μM and the label was added during the last 16 hours

15   of a 5-day culture. The peptides were synthesized
using standard Fmoc chemistry and purified by reverse
phase HPLC (Advanced Chemtech). The sequence of
stimulatory peptides are shown below in Table 9.

TABLE 9

20

Peptide Number	GAD Region	Amino Acid Sequence
6	78-97	KPCSCSKVDVNYAFLHATDL
17	247-266	NMYAMMIARFKMFPEVKEKG
23	335-356	TAGTTVYGAFDPLLAVADICKK
32	479-498	EYLYNIIKNREGYEMVFDGK
34	509-528	IPPSLRYLEDNEERMSRLSK
35	524-543	SRLSKVAPVIKARMMEYGTT
36	539-558	EYGTTMVSYQPLGDKVNFFR
38	566-585	ATHQDIDFLIEEIERLGQDL

Murine and human GAD_{65} are 95% identical at the

35   amino acid level (555/585) and are 98% conserved, with
m st of the differences localized near th ir N-termini.
The underlined amino acid in the stimulatory peptide

- 64 -

sequences above are conservatively substituted in
murine GAD$_{65}$. In separate experiments, the murine form
of key peptides (#17 and #34) were tested and produced
similar results.

5   As shown in Figure 10, peptides that triggered
stimulation indices >3 are indicated as black bars.
These peptides did not induce proliferation in T-cells
from NOD mice <3 or >16 weeks in age, or from control
(BALB/c x NOD)F1 mice (data not shown). The data are
10   represented as the mean SI ± standard error calculated
from 3-6 individual mice tested twice in each age
group. Characteristic results for peptide induced
blastogenesis in individual mice are shown in Table 6.
The first detectable response, at 4 weeks of age, was
15   confined to the carboxy-terminal region of GAD, and
involved two adjacent peptides (aa 509-528 and 524-543,
peptides #34 and #35, respectively, Figure 10a). At 5
weeks of age, responses to an additional determinant
(aa 247-266, peptide #17, which contains a region of
20   sequence similarity with Coxsackievirus (Kaufman, et
al., J. Clin. Invest., 89:283-292, 1992) (Figure 10b)
were regularly recorded. During the next two weeks,
responses to peptide #17 (aa247-266) increased and T-
cell autoimmunity spread to two additional peptides at
25   the carboxy terminus (aa 479-498 and 539-558; peptides
#32 and #36 respectively, Figure 10c). Subsequently,
reactivity to the GAD peptides declined (data not
shown), paralleling the loss of response to the whole
protein (Figure 8). It is unclear why the initial T-
30   cell response to βCAs fades in NOD mice. Possible
explanations include: a) immune regulatory mechanisms;
b) exhaustion of the response due to the continuous
stimulation by the endogenous antigen; and c) induction
of anergy in specific T-cells owing to their
35   recognition of the autoantig n on "n n professi nal"
antig n pr senting cells such as the β cells themselves
(Markmann, et al., Nature, 336:476-479, 1988).

- 65 -

        The gradual diversification of the primed
autoreactive T-cell repertoire that was observ d in
this naturally occurring autoimmune disease parallels
the shifts in T-cell recognition recently observed in
experimentally induced autoimmunity to the CNS where
autoreactivity spreads both intra- and intermolecularly
among CNS proteins (Lehmannn, et al., Nature, 358:155-
157, 1992; Perry, et al., J. Neuroimmunol., 33:7-15,
1991; Watanabe, et al.,Nature, 305:150-153, 1983;
Liebert, et al., J. Neuroimmunol.,17:103-118, 1988).
Apparently, lymphokine secretion by the first wave of
autoantigen specific T-cells in the target organ
results in up-regulation of antigen presentation and
creates a microenvironment that favors priming of
additional autoreactive T-cells (Lehmann, et al.,
Immunol. Today, 14:203-208, 1993; Sarvetnick, et al.,
Nature, 346:844-847, 1990; Heath, et al., Nature,
359:547-549, 1992). Since hsp reactive CD4+ T-cells
are capable of inducing IDDM (Elias, et al., Proc.
Natl. Acad. Sci., 87:1576-1580, 1990; Elias, et al.,
Proc. Natl. Acad. Sci., 88:3088-3091, 1991), their
recruitment into the activated T-cell pool, along with
T-cells reactive to other βCAs, probably reflects an
amplificatory cascade that eventually leads to β cell
destruction.

        In summary, the data above establish GAD as a
critical target antigen in the pathogenesis of IDDM in
NOD mice. The results show that T-cell responses to
βCAs diversify both intramolecularly and
intermolecularly as the disease progresses, consistent
with a dynamic autoimmune repertoire (Lehmann, et al.,
Immunol. Today, 14:203-208, 1993). However,
interference with the early autoreactive T-cell
population can prevent the recruitment of additional
autoantigens int the primed repertoire thereby halting
a cascade of autoimmune responses that eventually leads
to β cell d struction. As a similar autoimmun

- 66 -

progr ssion is also likely to occur during the
development of human IDDM (Palmer, J.P., Predicting
IDDM, *Diabetes Reviews*, 1:14-115, 1993; Atkinson, *et
al.*, *Lancet*, 339:458-459, 1992), these findings suggest
that peptide-based immunotherapeutic agents would be
useful in predicting and ameliorating human IDDM.

EXAMPLE 9

AUTOANTIBODY REACTIVITY WITH GAD FRAGMENTS

This example describes a study which examined the
variability in recognition of epitopes in human GAD_{65}
polypeptides by IDDM autoantibodies in sera of human
patients.

Portions of human GAD_{65} cDNA were amplified by the
polymerase chain reaction (PCR; Saiki, *et al.*, *Science*,
239:487, 1988) to produce DNA segments encoding three
polypeptide segments: amino acid residues 1-224
(segment A); 224-398 (segment B); and 398-585 (segment
C). Each construct also contained a T_7 promoter, a
consensus sequence for the initiation of translation
and an initiating methionine codon (Korak, M., *J. Cell
Biol.*, 108:229, 1989). Each PCR product was then
trascribed *in vitro* with T_7 RNA polymerase and
translated *in vitro* in a rabbit reticulocyte cell-free
system in the presence of ^{35}S-methionine, using
conditions recommended by the supplier (Amersham Corp.,
Arlington Heights, IL). Each test serum (30 μl) was
incubated with the resulting ^{35}S labeled-polypeptides.
The bound peptides were isolated with PAS and analyzed
by SDS-PAGE in 12% polyacrylamide and autoradiography.

TABLE 10

IDDM PATIENT SERA REACTIVITY WITH GAD SEGMENTS

	PATIENT	A	B	C
5	Control (N=7)	-	-	-
	052	-	+	+
	723	-	-	-
	705	-	+	+
10	UC2	-	+	+
	N.L.	-	-	-
	L.I.	-	-	-
	T.L.	-	-	-
	P.T.	-	+	-
15	J.D.	-	-	-
	B.Y.	-	+	+
	M.C.	-	-	-
	R.S.	-	-	-
	K.O.	-	-	-
20	T.B.	-	-	-
	S.M.	-	-	-
	A.W.	-	+	-
	J.B.	-	+	+
	J.A.	-	-	-
25	P.C.	-	+	+
	L.R.	-	-	-
	J.M.	-	+	-
	G.A.	-	-	-

- 68 -

As shown in Table 10, none of the specimens had
d t ctable lev ls of antibodies to the amino terminal
third (segment A) of GAD whereas 9 patients (41%) had
antibodies reactive with the middle third (segment B)
5   and 6 patients (27%) had antibodies to the carboxyl-
terminal third (segment C) of GAD.

EXAMPLE 10

PREDICTION OF INCIPIENT IDDM BY GAD
EPITOPE RECOGNITION PATTERN

10   The increasing likelihood of an IDDM interventive
therapy and the (recently acknowledged) benefits of
managed glucose homeostasis in preventing IDDM
associated complications makes the early detection of β
cell autoimmunity before clinical IDDM onset and in
15   NIDDM patients (10% of whom eventually convert to IDDM)
a crucial goal. Autoanitbodies to GAD may provide the
earliest and most reliable marker of impending IDDM
among the molecularly defined IDDM associated
autoantigens. To determine whether GAD peptides will
20   bind to IDDM associated autoantibodies the following
study was conducted.

A set of peptides (20-23 amino acids in length,
with 5 aa overlaps) that span the human GAD65 molecule
were synthesized to determine whether sera from most
25   individuals at risk, pre-IDDM and with IDDM (in
contrast to healthy controls) do in fact produce
antibodies that differentially recognize GAD65 linear
epitopes distributed throughout the molecule.

Patient sera and most control sera were those used
30   in a previous study (Kaufman, et al., J. Clin.
Investigation, supra) All samples were coded and tested
in a blind manner. Peptides were synthesized using an
automatic instrument (Applied Biosystems, Foster City,
CA) and standard conditions. Peptides were dissolved
35   in 60 mM s dium bicarbonate buffer (pH 9.6) at 20 ug/ml
and 100 ul of each was add d to duplicated wells of a
96 well Nunc-Immuno Plate. P ptid s were allow d to

- 69 -

bind at 4°C overnight. The plates w re then washed
three times with PBS + 0.1% Tween 20 (wash buffer),
after which the plates were pre-absorbed with 3% BSA in
sodium bicarbonate buffer for 0.5 hours at 37°C, or at

5   room temperature overnight. The plates were then
washed 5 times with the above wash buffer. 100 ul of
serum at a 1/300 dilution in PBS + 0.1% Tween 20 and 1%
BSA was added to each well and antibodies were allowed
to bind for 1 hour at 37°C. The plates were washed 5

10   times with wash buffer. 100 ul of a 1/600 dilution of
HRP-goat anti-human IgG (BRL, Gaithersberg, MD) was
added to each well and allowed to bind for 1 hour at
37°C. The plates were then washed 7 times and 100 ul
of substrate buffer was added to each well for 30

15   minutes at room temperature. The color development was
measured at 410 nm using an ELISA plate reader (ICN,
Biomedicals, Costa Mesa, CA). Positive sera were
defined as: OD_{410} of the sample/negative control ≥ 3.0.

20   The data shown in Table 11 establish that a number of
GAD peptides were recognized by patients previously
shown to be 64K positive, but not by control sera.
Each patient showed a different pattern of GAD epitope
recognition. Peptides 20, 21 and 25, were each

25   recognized by 6/8 patients, and none of the controls--
with the exception of peptide 25 which was recognized
by 1 out of 13 controls. Based on immunoreactivity to
2 of these peptides (#20 and 21) 7/8 (88%) of the
patients (and none of the controls) could be identified

30   as possessing GAD autoantibodies. Peptides 3, 6, 22,
25 and 37 were each recognized by only 25-37% of the
patients (and none of the control sera), but taken
together, 75% of the patients recognized at least one
of these. Peptides 5, 9 and 24 were often positive for

35   immun reactivity by both control and patient sera.
        This level of s nsitivity is comparable to the best
curr ntly available assays using whole GAD65 purifi d

from brain or recombinant organisms. Besides avoiding laborious antig n purification, peptide based autoantibody screening, together with PCR based HLA typing, may reveal epitope recognition patterns associated with progression or lack of progression to IDDM and its associated complications. Individuals determined to be at high risk could then consider therapeutic intervention.

It should also be noted that the GAD peptides recognized by autoantibodies were different from those recognized by NOD GAD reactive T cells in Example 6.

TABLE 11

EPITOPE RECOGNITION OF HUMAN GAD65 PEPTIDES

PEPTIDE

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
Controls																																						
A.P.					x				x															x														
P.T.					x	x																		x														
5748					x				b															x		b				b								
5620					x				b															x							b							
4380					x				b															x	x							b						
S.M.					x				x															x	b		b					b						
FA8																																						
FA12					b																b			x	b													
1688					b																			x														
FA9					x																			x														
H.B					b																			x														
FA2					x																			x														
FA11					x																			x														
IDDM									2																1													
At Risk																																						
052			b		x	x			b					b	b		b		x	x				x				b	b x		b							
825					x						x													x	b													
356					x				x												x			x	x													
L.I.			b		x			x			b									b				x								b						

PEPTIDE

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
Pre-IDDM																																						
J.A.			X		X				X											X	X			X	X													
723			X		X		b	b	b											X	X			X	X				X						X		X	
P.T. (1)			b		b		b	b	b	b										b	b			X	b				b			b		b				
P.T. (2)					X	b		b	b	b										X				X	X													
J.B. (1)			b		X		b	b	b	b	b									b	b			X	b													
J.B. (2)			X		X						X													X	X													
R.S. (1)					X																			X														
R.S. (2)					X																			X														
B.Y. (1)			b		b	b	b	b	b	b	b									b	b			X	b				b			b	b	b				
B.Y. (2)					X		b	b		b	b									X	X			X	X		X		X			X	X	X				X
At Onset																																						
705			X		X			X											1	X	X	X		X	X				X						X	X	X	
S.H.		b	b		X															X	X	X		X	X		X					b		b				b
291			X	X	X	X	b													X	X	X		X	X		X		X			X		X				X
048									X											X	X			X	X				X					X				1
Total			5	1	2	2	2	2	3	2	2								1	7	7	2		8	8		1		4			1		1	1	1	2	1

X = positive for immunoreactivity as defined by OD/background≥3.0
30b = borderline response (OD/background =2.5-2.9).

- 73 -

EXAMPLE 11

GAD IMMUNIZATION PROTECTS NOD MICE FROM IDDM

The availability of cDNAs encoding GAD65 allows the
testing of this molecule in new interventive therapies
designed to interfere with GAD-specific T cells. Tests
were conducted to examine the ability of GAD65
immunization to protect NOD mice at 8 weeks of age, a
time at which T cell responses to a number of b cell
antigens and insulitis is already well established. If
GAD immunotherapy was effective at this stage, it would
hold promise for treatment in humans in which the
autoimmune process has already been established.

METHODS

Antigens

An IPTG inducible T7 expression vector was used to
express both human GAD65 and *E. coli* b galactosidase
(β-gal). In IPTG induced recombinant *E. coli*, GAD and
β-gal constitute about 10-20% of the total bacterial
protein. However, almost all of the GAD was in
inclusion bodies, which could be isolated and
extensively washed to obtain material that is about 80%
GAD. We then did affinity purifications of GAD and β-
gal on the basis of a hexa-histidine "tag" which was
attached to GAD during the subcloning process. These
extra histidine residues allow the rapid affinity
purification (Novagen) of GAD by metal affinity
chromatography (Hochuli, *et al.*, Bio Technology,
6:1321-1325, 1988). The inclusion body material is
solubilized in 6M guanidine hydrochloride (GHCL), 10mm
β-mercaptoethanol and 1% triton X-100. After binding
to the column, the column was extensively washed with
GHCL and 8M urea in phosphate buffers. Only the
central peak GAD fraction was utilized for subsequent
studies. Human GAD65 shares 96% amino acid sequence
identity with murine GAD65, with most of the amino acid
differ nces being conservativ substitutions.

- 74 -

The GAD preparation appeared to be free of
immunologically detectable contaminants. It also
appeared to be free of bacterial contaminants on
overloaded silver stained gels. Analysis by a national
5   reference laboratory found $\langle$0.06ng LPS/ug GAD. Human
GAD65 did not induce T cell proliferation in $\langle$4 or $\rangle$16
week old NOD or control BALB/c or (NOD/BALB/c) F1
spleen cells. The results using synthetic GAD peptides
(Figure 10) precisely parallel the data using whole
10   recombinant GAD (Figure 8). Other antigens described
herein elsewhere that are not involved in IDDM (such as
the beta galactosidase) did not induce NOD T cell
responses. After immunizing mice with GAD, we were
unable to detect cross reactive T cell responses in
15   recall experiments with other proteins that were
purified from recombinant *E. coli* by the same metal
affinity chromatography procedure. Amino acid sequence
analysis of GAD and β-gal each gave a single expected
amino acid N-terminal sequence. If there had been
20   appreciable endotoxins, heat shock proteins, or other
contaminants present in the GAD preparation, spleen,
PBMC (Atkinson, *et al.*), and T cell proliferation
responses that were not disease specific would have
been expected.
25   Breeder mice were purchased from Taconic Farms and
housed under specific pathogen-free conditions. Only
female NOD mice were used in this study. The average
age of IDDM onset in unrelated females in the colony
was 22 weeks. Insulitis is generally observed
30   beginning at 4 weeks of age. T cell responses to GAD,
HSP, CPH were found by 6 weeks in age. The incidence
of IDDM in female mice is 70-90% by one year of age.
Immunizations
At 8 weeks of age, 25 ug GAD or control β-gal. was
35   injected intraperitoneally (ip) in 100 ul of incomplete
Freunds adjuvant (IFA). Because there may be a
r quir ment for continual antigen pres ntation

- 75 -

(Ramsdell, *et al*., Science, <u>257</u>:1130-1133, 1992) mice
were tr ated again every 6 weeks. Urine glucose levels
were monitored twice weekly. After observing above
normal glucose in urea, blood glucose levels were
5   monitored twice weekly. Two consecutive blood glucose
level readings of 300 mg/ml was considered as IDDM
onset, after which the mice were sacrificed and spleen
cells were tested as described above in Example 6 for
evidence of spleen cell proliferation.
10   Immunization of 8 week old NOD mice produced a
clear delay in the onset of IDDM compared to control β-
gal immunized mice (Fig. 11). While two of the GAD
immunized mice (open circles) developed IDDM at about
the normal age of onset (20 weeks), the other 8 GAD
15   immunized mice showed no signs of hyperglycemia until
36 weeks in age. Four of the GAD treated mice
developed IDDM between 37 and 40 weeks in age. Four of
the GAD treated mice currently remain disease free (at
52 weeks of age). In contrast, the majority of β-gal
20   injected mice (closed circles) had hyperglycemia by 22
weeks of age and 6/10 developed IDDM by 27 weeks in
age. At 52 weeks of age, 2 of the β-gal treated mice
remain disease free. This experiment shows that GAD
immunization significantly delayed ($<$0.02) or prevented
25   diabetes of NOD mice in which β cell autoimmunity has
already significantly progressed.
           β cell autoimmunity is already well established at
8 weeks of age, and it is likely to also be in
·individuals determined to be at risk for IDDM on the
30   basis of circulating autoantibodies. Although the
mechanism of this protection is not clear, periodic
injections of GAD have a profound moderating effect on
the induction of disease.

- 76 -

TABLE 11

AMINO ACID SEQUENCES FOR GAD65

	1	MASPGSGFWSFGSEDGSGDS
5	2	GSGDSENPGTARAWCQVAQKFTG
	3	QKFTGGIGIGNKLCALLYGD
	4	LLYGDAEKPAESGGSQPPRA
	5	QPPRAAARKAACACDQKPCSC
	6	KPCSCSKVDVNYAFLHATDL
10	7	HATDLLPACDGERPTLAFLQ
	8	LAFLQDVMNILLQYVVKSFDRS
	9	SFDRSTKVIDFHYPNELLQE
	10	ELLQEYNWELADQPQNLEEILM
	11	EEILMHCQTTLKYAIKTGHP
15	12	KYGHPRYFNQLSTGLDMVGL
	13	DMVGLAADWLTSTANTNMFT
	14	TNMFTYEIAPVFVLLEYVTL
	15	EYVTLKKMREIIGWPGGSGD
	16	GGSGDGIFSPGGAISNMYAM
20	17	NMYAMMIARFKMFPEVKEKG
	18	PEVKEKGMAALPRLIAFTSE
	19	AFTSEHSHFSLKKGAAALGI
	20	AALGIGTDSVILIKCDERGK
	21	DERGKMIPSDLERRILEAKQ
25	22	LEAKQKGFVPFLVSATAGTT
	23	TAGTTVYGAFDPLLAVADICKK
	24	DICKKYKIWMHVDAAWGGGLLMS
	25	GLLMSRKHKWKLSGVERANS
	26	ERANSVTWNPHKMMGVPLQC
30	27	VPLQCSALLVREEGLMQNCNQ
	28	QNCNQMHASYLFQQDKHYDL
	29	KHYDLSYDTGDKALQCGRHV
	30	CGRHVDVFKLWLMWRAKGTTG
	31	KGTTGFEAHVDKCLELAEYLYN
35	32	EYLYNIIKNREGYEMVFDGK
	33	VFDGKPQHTNVCFWYIPPSL
	34	IPPSLRTLEDNEERMSRLSK

- 77 -

```
35   SRLSKVAPVIKARMMEYGTT

36   EYGTTTMVSYQPLGDKVNFFR

37   VNFFRMVISNPAATHQDIDF

38   ATHQDIDFLIEEIERLGQDL
```

5

The invention now being fully described, it will be
apparent to one of ordinary skill in the art that many
changes and modifications can be made without departing
10   from the scope of the invention.

- 78 -

CLAIMS

1.   A polypeptide consisting essentially of an amino
acid sequence selected from the group consisting
of:

KPCSCSKVDVNYAFLHATDL;

NMYAMMIARFKMFPEVKEKG;

TAGTTVYGAFDPLLAVADICKK;

EYLYNIIKNREGYEMVFDGK;

IPPSLRYLEDNEERMSRLSK;

SRLSKVAPVIKARMMEYGTT;

EYGTTMVSYQPLGDKVNFFR;

ATHQDIDFLIEEIERLGQDL;

LAFLQDVMNILLQYVVKSFDRS;

EEILMHCQTTLKYAIKTGHP;

DERGKMIPSKLERRILEAKQ;

KHYDLSYDTGDKALQCGRHV;

AALGIGTDSVILIKCDERGK;

GLLMSRKHKWKLSGVERANS;

LEAKQKGFVPFLVSATAGTT; and

VNFFRMVISNPAATHQDIDF.

2.   An isolated polynucleotide sequence encoding a
polypeptide of claim 1.

3.   The polynucleotide of claim 2 which is DNA.

4.   A method for detecting autoantibody to GAD in a
patient specimen which comprises contacting the
specimen with a GAD fragment and determining
whether autoantibody binds to the fragment.

5.   The method of claim 4, wherein the fragment is a
polypeptide of at least about 16 amino acids in
length and is selected from the amino acid
sequence from about amino acid 224 to about amino
acid 585 of GAD_{65}.

6.   The method of claim 4, wherein the fragment is a
polypeptide of claim 1.

7.   The method of claim 4, wherein the specimen is
blood.

- 79 -

8.   The method of claim 4, wherein the peptide is
        detectably lab l d.

9.   The method of claim 8, wherein the detectable
        label is selected from the group consisting of a
        radioisotope, a fluorescent compound, a colloidal
        metal, a chemiluminescent compound, a
        bioluminescent compound, a phosphorescent
        compound, and an enzyme.

10.   The method of claim 4, wherein the peptide is
        bound to a solid phase.

11.   A monoclonal antibody to the peptide of claim 1.

12.   A hybridoma cell line capable of producing the
        monoclonal antibody of claim 9.

13.   A vector containing the polynucleotide of claim
        2.

14.   A host cell transformed with the polynucleotide
        of claim 2.

15.   A method for ameliorating a GAD associated
        autoimmune disorder in a patient having or at
        risk of having the disorder, which comprises
        administering to the patient a therapeutically
        effective amount of whole GAD_{65} or a
        therapeutically effective fragment thereof.

16.   The method of claim 15, wherein the
        therapeutically effective fragment is a
        polypeptide of at least about 16 amino acids in
        length and is selected from the amino acid
        sequence from about amino acid 224 to about amino
        acid 585 of GAD_{65}.

- 80 -

17. The method of claim 15, wherein the
     therapeutically effective fragment is selected
     from the group consisting of:
             KPCSCSKVDVNYAFLHATDL;
             NMYAMMIARFKMFPEVKEKG;
             TAGTTVYGAFDPLLAVADICKK;
             EYLYNIIKNREGYEMVFDGK;
             IPPSLRYLEDNEERMSRLSK;
             SRLSKVAPVIKARMMEYGTT;
             EYGTTMVSYQPLGDKVNFFR; and
             ATHQDIDFLIEEIERLGQDL.

18. The method of claim 15, wherein the
     therapeutically effective fragment is selected
     from the group consisting of:
             KPCSCSKVDVNYAFLHATDL;
             LAFLQDVMNILLQYVVKSFDRS;
             EEILMHCQTTLKYAIKTGHP;
             DERGKMIPSKLERRILEAKQ;
             KHYDLSYDTGDKALQCGRHV;
             AALGIGTDSVILIKCDERGK;
             GLLMSRKHKWKLSGVERANS;
             LEAKQKGFVPFLVSATAGTT; and
             VNFFRMVISNPAATHQDIDF.

19. The method of claim 16, wherein the disorder is
     IDDM.

20. The method of claim 16, wherein the disorder is
     stiff man disease.

21. The method of claim 16, wherein the
     administration is enteral.

22. The method of claim 21, wherein the enteral
     administration is oral.

23. The method of claim 15 or 16, wherein the
     administration is parenteral.

24. The method of claim 23, wherein the parenteral
     administration is by subcutaneous, intramuscular,
     intraperitoneal, intracavity, transdermal,
     intranasal, or intravenous inj ction.

25.   The method of claim 15, wherein said
        administration is at a dosage of about 0.01
        mg/kg/dose to about 2000 mg/kg/dose.

26.   The method of claim 15, wherein the polypeptide
5   is therapeutically labeled.

27.   The method of claim 26, wherein the therapeutic
        label is selected from the group consisting of a
        radioisotope, a drug, a lectin, and a toxin.

28.   A method for detecting the status of a GAD
10   associated autoimmune disorder in a patient which
        comprises contacting a T-cell of the patient with
        at least one polypeptide of claim 1 and detecting
        the response of the T-cell to the peptide.

15 29.   The method of claim 26, wherein the response of
        the T-cell is stimulation.

30.   The method of claim 27, wherein the stimulation
        is measured by cellular uptake of radiolabelled
        nucleoside.

20 31.   The method of claim 30, wherein the nucleoside is
        ^{3}H thymidine.

32.   A kit useful for the detection of antibody to the
        polypeptide of claim 1 in a specimen suspected of
        containing such antibody, the kit comprising
25   carrier means compartmentalized to receive in
        close confinement therein one or more containers
        comprising a container containing the polypeptide
        of claim 1.

33.   A kit useful for determining the status of a GAD
30   associated disorder in a specimen of a patient,
        the kit comprising carrier means
        compartmentalized to receive in close confinement
        therein one or more containers comprising a
        container containing a polypeptide of claim 1.

35

34.   A method for detecting a GAD associated
       autoimmune disorder in a patient having or at
       risk of having the disorder, which comprises
       administering to the patient a diagnostically
5   effective amount of a diagnostically effective
       fragment of GAD$_{65}$.

35.   The method of claim 34, wherein the
       diagnostically effective fragment is a
       polypeptide of at least about 16 amino acids in
10   length and is selected from the amino acid
       sequence from about amino acid 34 to 573 of GAD$_{65}$.

36.   The method of claim 35, wherein the
       diagnostically effective fragment is selected
       from the group consisting of:
15   KPCSCSKVDVNYAFLHATDL;
                LAFLQDVMNILLQYVVKSFDRS;
                EEILMHCQTTLKYAIKTGHP;
                DERGKMIPSKLERRILEAKQ;
                KHYDLSYDTGDKALQCGRHV;
20   AALGIGTDSVILIKCDERGK;
                GLLMSRKHKWKLSGVERANS;
                LEAKQKGFVPFLVSATAGTT; and
                VNFFRMVISNPAATHQDIDF.

37.   The method of claim 35, wherein the disorder is
25   IDDM.

38.   The method of claim 35, wherein the disorder is
       stiff man disease.

feline (from cDNA)   YEIAPVFVLMEQITLKKMREIVGWSSKDGDGIFSPGGAISNMYSIMAARYKFFPEVKTKG

rat (from peptide)   YEIAPVFVLLEYV-----REIIGWPGGS-DGIFSPGGAISN-YAMLIARYKMFPEVKEKG

FIG. 1

```
GGGCGGTGCGGGGGTCGGAGCCGGAAGCAGCTTGCCCGCGAGCCACTCGGAGGCGACCAGCGCCA
                    10                  30                  50

 M   A   S   P   G   S   G   F   W   S   F   G   S   E   D   G
GACTAGCAGAACCCATGGCCATCTCCGGGCTCTGGTCCTTCGGATCTGAAGATG
        70                  90                  110

 S   G   D   P   E   N   P   G   T   A   R   A   W   C   Q   V   A   Q   K   F
GCTCTGGGGATCCTGAGAACCCGGGAACAGCAGCCTGGTGCCCAGTGGCCAAAAGT
            130                 150                 170

 T   G   G   I   G   N   K   L   C   A   L   L   Y   G   D   S   E   K   P   A
TCACGGGGGCATCGGGAAACAAGCTATGCGCTCTGCTCTACGGAGACTCTGAGAAGCCAG
            190                 210                 230

 E   S   G   G   S   V   T   S   R   A   T   R   K   V   A   C   T   C   D
CAGAGAGCGGCGGGAGCGTGACCTCGCGGGCGCCACTCGGAAGGTCGCCTGCACCTGTG
            250                 270                 290

 Q   K   P   C   S   C   P   K   G   D   V   N   Y   A   L   L   H   A   T   D
ACCAAAAACCCTGCAGCTGCCCCAAAGGAGATGTCAATTATGCACTTCTCCACGCAACAG
            310                 330                 350

 L   L   P   A   C   E   G   E   R   P   T   L   A   F   L   Q   D   V   M   N
ACCTGCTGCCAGCCTGTGAAGGAGAAAGGCCCACTCTCGCATTTCTGCAAGATGTAATGA
            370                 390                 410

 I   L   L   Q   Y   V   K   S   F   D   R   S   T   K   V   I   D   F   H
ACATTTTGCTTCAGTGACGTGGTGAAAAGTTTGATAGATCAACTAAAGTGATTGATTTCC
            430                 450                 470

 Y   P   N   E   L   L   Q   E   Y   N   W   E   L   A   D   Q   P   Q   N   L
ATTACCCCAATGAGCTTCTTCAAGAGTATAATTGGGAATTGGCAGACCAACCGCAAAATC
            490                 510                 530

 E   E   I   L   T   H   C   Q   T   T   L   K   Y   A   I   K   T   G   H   P
TGGAGGAAATTTTGACGCACTGCCAAACAACTCTAAAAATATGCGATTAAAACAGGGCATC
            550                 570                 590

 R   Y   F   N   Q   L   S   T   G   L   D   M   V   G   L   A   D   W   L
CCCGATATTTTAATCAGTCAGTCTGTCTACCGGGATTGGGATATGGTTGGATTAGCAGATTGGT
            610                 630                 650

 T   S   T   A   N   T   M   F   T   Y   E   I   A   P   V   F   V   L   L
TGACATCAACAGCAAACACGAACATGTTTACCTATGAGATCGCCCCTGTATTTGTACTAC
```

FIG. 2A

```
                670                    690                    710
        E  Y  V  T  L  K  K  M  R  E  I  I  G  W  P  G  G  S  G  D
TGGAATATGTGACACTAAAGAAGATGAGGGAAATCATTGGCTGGCCAGGAGGCTCTGGCG

                730                    750                    770
        G  I  F  S  P  G  G  A  I  S  N  M  Y  A  M  L  I  A  R  Y
ATGGAATCTTTTCTCCTGGTGGTGCCATCTCCAACATGTACGCCATGCTCATTGCCCGCT

                790                    810                    830
        K  M  F  P  E  V  K  E  K  G  M  A  A  V  P  R  L  I  A  F
ATAAGATGTTTCCAGAAGTCAAGGAGAAAGGGGATGGCGGCGGTGCCCAGGCTCATCGCAT

                850                    870                    890
        T  S  E  H  S  H  F  S  L  K  K  G  A  A  L  G  I  G  T
TCACGTCAGAGCATAGTCACTTTTCTCTCAAGAAGGGAGCTGCAGCCTTGGGATCGGAA

                910                    930                    950
        D  S  V  I  L  I  K  C  D  E  R  G  K  M  I  P  S  D  L  E
CAGACAGCGTGATTCTGATTAAATGTGATGAGAGGGGAAAATGATCCCATCTGACCTTG

                970                    990                    1010
        R  R  I  L  E  V  K  Q  K  G  F  V  P  F  L  V  S  A  T  A
AAAGAAGAATCCTTGAAGTAGTCAAACAGAAAGGATTTGTTCCTTTCCTGGTGAGTGCCACAG

                                       1030                   1050
        R  R  I  L  E  V  K  Q  K  G  F  V  P  F  L  V  S  A  T  A
AAAGAAGAATCCTTGAAGTAGTCAAACAGAAAGGATTTGTTCCTTTCCTGGTGAGTGCCACAG

                1070                   1090                   1110
        G  T  T  V  Y  G  A  F  D  P  L  L  A  V  A  D  I  C  K  K
CTGGAACCACTGTGTACGGGGCTTTTGATCCTCTCCTTGGCTGTAGCTGACATCTGCAAAA

                1130                   1150                   1170
        Y  K  I  W  M  H  V  D  A  A  W  G  G  G  L  L  M  S  R  K
AATATAAGATCTGGATGCATGTGGATGCTGCTTGGGGGTGGAGGGTTACTGATGTCTCGGA

                1190                   1210                   1230
        H  K  W  K  L  N  G  V  E  R  A  N  S  V  T  W  N  P  H  K
AACACAAGTGGAAGCTGAACGGTGTGGAGGGCCAACTCTGTGACATGGAATCCCCACA

                1250
        M  M  G  V  P  L  Q  C  S  A  L  L  V  R  E  E  G  L  M  Q
AGATGATGGGTGTCCCCCTTGCAATGTTCGGCTCTCCTGGTCAGAGAGAGGAGGGACTGATGC
```

FIG. 2B

```
            1270                      1290                      1310
     S   C   N   Q   M   H   A   S   Y   L   F   Q   Q   D   K   H   Y   D   L   S
AGAGCTGCAACCAGATGATGCATGCTTCCTACCTCTTTCAGCAAGATAAGCACTATGACCTGT
            1330                      1350                      1370
     Y   D   T   G   D   K   A   L   Q   C   G   R   H   V   D   V   F   K   L   W
CCTATGACACGGGAGACAAGGCCTTGCAGTGTGGACGCCACGTCGATGTCTTTAAATTAT
            1390                      1410                      1430
     L   M   W   R   A   K   G   T   G   F   E   A   H   I   D   K   C   L   E
GGCTCATGTGGAGAGCAAAGGGGACTACTGGATTTGAAGCTCACATTGATAAGTGTTTGG
            1450                      1470                      1490
     L   A   E   Y   L   Y   N   I   I   K   N   R   E   G   Y   E   M   V   F   D
AGCTGGCAGAGTATTTATACAATATCATTAAAAACCGAGAAGGATATGAAATGGTGTTCG
            1510                      1530                      1550
     G   K   P   Q   H   T   N   V   C   F   W   F   V   P   P   S   L   R   V   L
ATGGGAAGCCTCAGCACACAAATGTCTGCTTCTGGTTTGTACCTCCTAGTTTGCGAGTTC
            1570                      1590                      1610
     E   D   N   E   E   R   M   S   R   L   S   K   V   A   P   V   I   K   A   R
TGGAAGACAATGAAGAGAGAATGAGCCGCCTCTCAAAGTGGGCGCCAGTGATTAAAGCCA
            1630                      1650                      1670
     M   M   E   Y   G   T   T   M   V   S   Y   Q   P   L   G   D   K   V   N   F
GAATGATGGAGTATGGGACCACAATGGTCAGCTACCAACCCTTAGGAGATAAGGTCAACT
            1690                      1710                      1730
     F   R   M   V   I   S   N   P   A   A   T   H   Q   D   I   D   F   L   I   E
TCTTCCGCATGGTCATCTCAAACCCTGCAGCAACATTCACCAAGACATTGACTTCCTCATTG
            1750                      1770                      1790
     E   I   E   R   L   G   Q   D   L   *
AAGAAATCGAACGCCTGGGACACAAGATTTGTAATCACTTGCTCACCAAACTTTCAGTTCT
            1810                      1830                      1850
CTAGGTAGACAGCTAAGTTGTCACAAACTGTGTAAATGTATTTGTAGTTTGTTCCAGAGT
            1870                      1890                      1910
AATTCTATTTCTATATCGTGGTGTCACAGTAGAGTCCAGTTTAAAA
            1930                      1950
```

FIG. 2C

```
                                      M   A   S
      agctcgcccgcagctcgcaggcgacctgctccagtctccaaagccgatggcatc
                                                      10

P   G   S   G   F   W   S   F   G   S   E   D   G   S   G   D   S   E   N   P
      tccgggctctggctttttggtcttttcggtcggaagatggctcggggattccgagaatcc
            30                      50                      70

G   T   A   R   W   C   Q   V   A   Q   K   F   T   G   G   I   G   N   K
      cggcacagcggcgagcctgccaagtgtgccaagttcacggcggcatcggaaacaa
                  90                     110

L   C   A   L   L   Y   G   D   A   E   K   P   A   E   S   G   G   S   Q   P
      actgtgccctgctcctacggagacgccgagaagccggagagcggcggagccaacc
            130                    150                    170

P   R   A   A   R   K   A   A   C   A   C   D   Q   K   P   C   S   C   S
      cccgcggccgcgcggaagccgccgacgtgccctgcgactgccagcctgctgctc
                  190          210                    230

K   V   D   V   N   Y   A   F   L   H   A   T   D   L   L   P   A   C   D   G
      caaagtggatgtcaactacgcgtttctcctccatgcaacagacctgctgccggtgtgatgg
            250                    270                    290

E   R   P   T   L   A   F   L   Q   D   V   M   N   I   L   L   Q   Y   V   V
      agaaaggcccactttggcgttctgcaagatgttatgaacattttacttcagtatgtggt
            370                    350                    330

K   S   F   D   R   S   T   K   V   I   D   F   H   Y   P   N   E   L   L   Q
      gaaaagtttcgatagatcaacaaagtgattgatttccattatcctaatgagcttctcca
            430                    450                    470

E   Y   N   W   E   L   A   D   Q   P   Q   N   L   E   E   I   L   M   H   C
      agaatatctcgataattggaattggcagcaacaccacaaaatttggaggaaatttgatgcattg
            490                    510                    530

Q   T   T   L   K   Y   A   I   K   T   G   H   P   R   Y   F   N   Q   L   S
      ccaaacacttctaaaatatgcaattaaaacagggcatcctagatactccatcaacttttc
                  550                    570                    590

T   G   L   D   M   V   G   L   A   D   W   L   T   S   T   A   N   T   N
      tactggtttggatatggttggattagcagcagactggctgacatcaacagcaaatactaa
```

FIG. 3A

```
             610            630            650
M   F   T   Y   E   I   A   P   V   F   V   L   L   E   Y   V   T   L   K   K
catgttcacctatgaaattgctccagtattgtgtcttttggaatatgtcacactaaagaa
             670            690            710
M   R   E   I   I   G   W   P   G   G   S   G   D   G   I   F   S   P   G   G
aatgagagaaatcattggctggccagggggctctggcgatgggatattttctcccggtgg
             730            750            770
A   I   S   N   M   Y   A   M   M   I   A   R   F   K   M   F   P   E   V   K
cgccatatctaacatgtatgccatgatgatcgcacgctttaagatgttcccagaagtcaa
             790            810            830
E   K   G   M   A   A   L   P   R   L   I   A   F   T   S   E   H   S   H   F
ggagaaaggaatggctgctcttcctcccaggctcattgccttcacgtctgaacatagtcatt
             850            870            890
S   L   K   K   G   A   A   A   L   G   I   G   T   D   S   V   I   L   I   K
ttctctcaagaagggagctgcagcctaggattggaacagacagcgtctgatcttgattaa
             910            930            950
C   D   E   R   G   K   M   I   P   S   D   L   E   R   R   I   L   E   A   K
atgtgatgagagaggaaaatgattccatctgatcttgaaagaagattcttgaagcaa
             970            990            1010
Q   K   G   F   V   P   F   L   V   S   A   T   A   G   T   T   V   Y   G   A
acagaaagggtttgttcctttcctgtgagtgccacagctgaaccaccgtgtacggagc
             1030           1050           1070
F   D   P   L   L   A   V   A   D   I   C   K   K   Y   K   I   W   M   H   V
atttgacccctctctagctgtcgctgacatttgcaaaaagtataagatctggatgcatgt
             1090           1110           1130
D   A   W   G   G   G   L   L   M   S   R   K   H   K   W   K   L   S   G
ggatgcagcttggggtggtgggggattactgatgtcccgaaaacaagtggaaactgagtgg
             1150           1170           1190
V   E   R   A   N   S   V   T   W   N   P   H   K   M   M   G   V   P   L   Q
cgtggagaggccaactctgtgacgtggaatccacacaagatgatgggagtcccttgca
```

FIG. 3B

```
     1210                1230                1250
C   S   A   L   L   V   R   E   E   G   L   M   Q   N   C   N   Q   M   H   A
gtgctctgctcctggttagagagaggattgatgcagaattgcaacaccaaatgcatgc
                      1270                1290
S   Y   L   F   Q   Q   D   K   H   Y   D   L   S   Y   D   T   G   D   K   A
ctcctacctctttcagcaagataaacattgacctgtcctatgacactggagacaaggc
                      1330                1350                1370
L   Q   C   G   R   H   V   D   V   F   K   L   W   L   M   W   R   A   K   G
cttacagtgcggacgccacgttgatgtttttaaactatggctgatgtggaggcaaaggg
                      1390                1410                1430
T   T   G   F   E   A   H   V   D   K   C   L   E   L   A   E   Y   L   Y   N
gactaccgggtttgaagcgcatgtttgataaatgttggcagagtattatacaa
                      1450                1470                1490
I   I   K   N   R   E   G   Y   E   M   V   F   D   G   K   P   Q   H   T   N
catcataaaaaccgagaagaggatatgagagatggtgtttgatgggaagcctcagcacacaaa
```

FIG. 3C

```
        1510             1530              1550
   V   C   F   W   Y   I   P   P   S   L   R   T   L   E   D   N   E   E   R   M
tgtctgctggtacattcctccaagcttgcgtactctggaagacaatgaagagagaat
        1570             1590              1610

   S   R   L   S   K   V   A   P   V   I   K   A   R   M   M   E   Y   G   T   T
gagtcgcctctcgaaggtgtgctccagtgctcagaatgattaaagccagaatgatggagtatggaaccac
        1630             1650              1670

   M   V   S   Y   Q   P   L   G   D   K   V   N   F   F   R   M   V   I   S   N
aatggtcagctaccaaccccttgggagacaaggtcaattcttccgcatgtcatctcaaa
        1690             1710              1730

   P   A   A   T   H   Q   D   I   D   F   L   I   E   E   I   E   R   L   G   Q
cccagcggcaactcaccagacaccaccagacttgactcctgattgaagaaatagaacgccttggaca
        1750             1770              1790

   D   L   *
agattatataataacccttgctcaccaagctgttccacttctctaggtagacaattaagttg
        1810             1830              1850

tcacaaactgtgtgaatgtagttgtattttgttcgttccaaagtaaatctatttctatattgtg
        1870             1890              1910

gtgtcaaagtagagtttaaaaaataaacaaacaaaaagacattgctcctttaaagtcctt
        1930             1950              1970

cttaagtttagaatacctctctaagaaattcgtgacaaaaggctatgtttctaatcaataag
        1990             2010              2030

gaaaagcttaaaaattgttatataactttccttacttttaatatagtgtgcaaagcaaac
        2050             2070              2090
```

FIG. 3D

GAP WEIGHT:	3.000	AVERAGE MATCH:	0.540
LENGTH WEIGHT:	0.100	AVERAGE MISMATCH:	-0.396
QUALITY:	856.2	LENGTH:	585
RATIO:	1.464	GAPS:	0
PERCENT SIMILARITY:	97.436	PERCENT IDENTITY:	96.068

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```
  1 MASPGSGFWSFGSEDGSGDPENPGTARAWCQVAQKFTGGIGNKLCALLYG 50
    ||||||||||||||||||||•|||||||||||||||||||||||||||||||
  1 MASPGSGFWSFGSEDGSGDSENPGTARAWCQVAQKFTGGIGNKLCALLYG 50

 51 DSEKPAESGGSVTSRAATRKVACTCDQKPCSCPKGDVNYALLHATDLLPA 100
    |•||||||||| ••|||•||•||•|||||||||•|•|||||:|||||||||
 51 DAEKPAESGGSQPPRAAARKAACACDQKPCSCSKVDVNYAFLHATDLLPA 100

101 CEGERPTLAFLQDVMNILLQYVVKSFDRSTKVIDFHYPNELLQEYNWELA 150
    |:|||||||||||||||||||||||||||||||||||||||||||||||||
101 CDGERPTLAFLQDVMNILLQYVVKSFDRSTKVIDFHYPNELLQEYNWELA 150

151 DQPQNLEEILTHCQTTLKYAIKTGHPRYFNQLSTGLDMVGLAADWLTSTA 200
    ||||||||||| |||||||||||||||||||||||||||||||||||||||
151 DQPQNLEEILMHCQTTLKYAIKTGHPRYFNQLSTGLDMVGLAADWLTSTA 200

201 NTNMFTYEIAPVFVLLEYVTLKKMREIIGWPGGSGDGIFSPGGAISNMYA 250
    |||||||||||||||||||||||||||||||||||||||||||||||||||
201 NTNMFTYEIAPVFVLLEYVTLKKMREIIGWPGGSGDGIFSPGGAISNMYA 250

251 MLIARYKMFPEVKEKGMAAVPRLIAFTSEHSHFSLKKGAAALGIGTDSVI 300
    |:|||:|||||||||||||:||||||||||||||||||||||||||||||
251 MMIARFKMFPEVKEKGMAALPRLIAFTSEHSHFSLKKGAAALGIGTDSVI 300

301 LIKCDERGKMIPSDLERRILEVKQKGFVPFLVSATAGTTVYGAFDPLLAV 350
    |||||||||||||||||||||•|||||||||||||||||||||||||||||
301 LIKCDERGKMIPSDLERRILEAKQKGFVPFLVSATAGTTVYGAFDPLLAV 350

351 ADICKKYKIWMHVDAAWGGGLLMSRKHKWKLNGVERANSVTWNPHKMMGV 400
    ||||||||||||||||||||||||||||||||||•||||||||||||||||
351 ADICKKYKIWMHVDAAWGGGLLMSRKHKWKLSGVERANSVTWNPHKMMGV 400
```

FIG.4A

```
401 PLQCSALLVREEGLMQSCNQMHASYLFQQDKHYDLSYDTGDKALQCGRHV 450
    |||||||||||||||||•||||||||||||||||||||||||||||||||
401 PLQCSALLVREEGLMQNCNQMHASYLFQQDKHYDLSYDTGDKALQCGRHV 450
            •        •           •        •        •

451 DVFKLWLMWRAKGTTGFEAHIDKCLELAEYLYNIIKNREGYEMVFDGKPQ 500
    ||||||||||||||||||||:|||||||||||||||||||||||||||||
451 DVFKLWLMWRAKGTTGFEAHVDKCLELAEYLYNIIKNREGYEMVFDGKPQ 500
            •        •           •        •        •

501 HTNVCFWFVPPSLRVLEDNEERMSRLSKVAPVIKARMMEYGTTMVSYQPL 550
    |||||||::||||||•||||||||||||||||||||||||||||||||||
501 HTNVCFWYIPPSLRTLEDNEERMSRLSKVAPVIKARMMEYGTTMVSYQPL 550
            •        •           •

551 GDKVNFFRMVISNPAATHQDIDFLIEEIERLGQDL 585
    ||||||||||||||||||||||||||||||||||
551 GDKVNFFRMVISNPAATHQDIDFLIEEIERLGQDL 585
```

FIG.4B

FIG.5

1 2   3 4

10.3 —   ⌐8.8   10.4 —
                  ⌐8.0
7.5 —
                  ⌐4.7   ⌐5.4
                  ⌐4.4   6.1 ⌐   ⌐5.1
3.3 —   5.3 ⌐   ⌐4.8
                  —3.1   ⌐4.4
                  —2.3   4.2 ⌐
2.2 ⌐   3.8 —
2.0 ⌐   3.5 —   —3.7
1.9 ⌐   3.0 —   ⌐3.4
1.6 —   2.8 ⌐
                             2.5 ⌐

0.9 —   —1.9
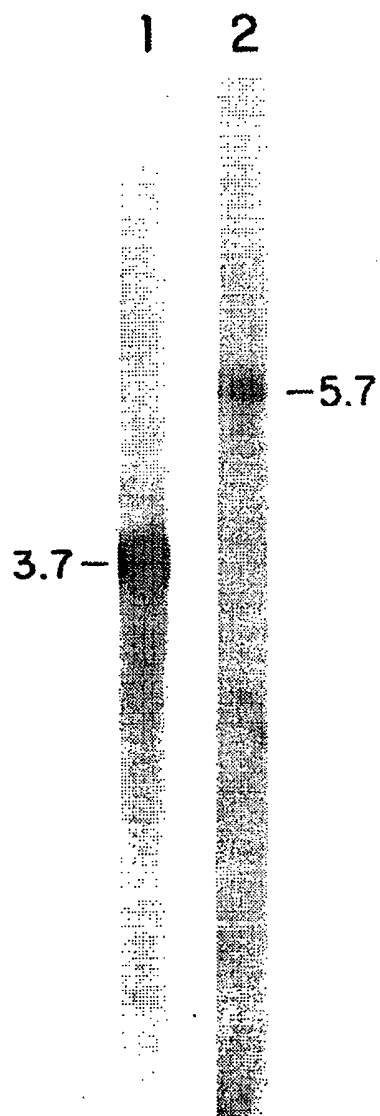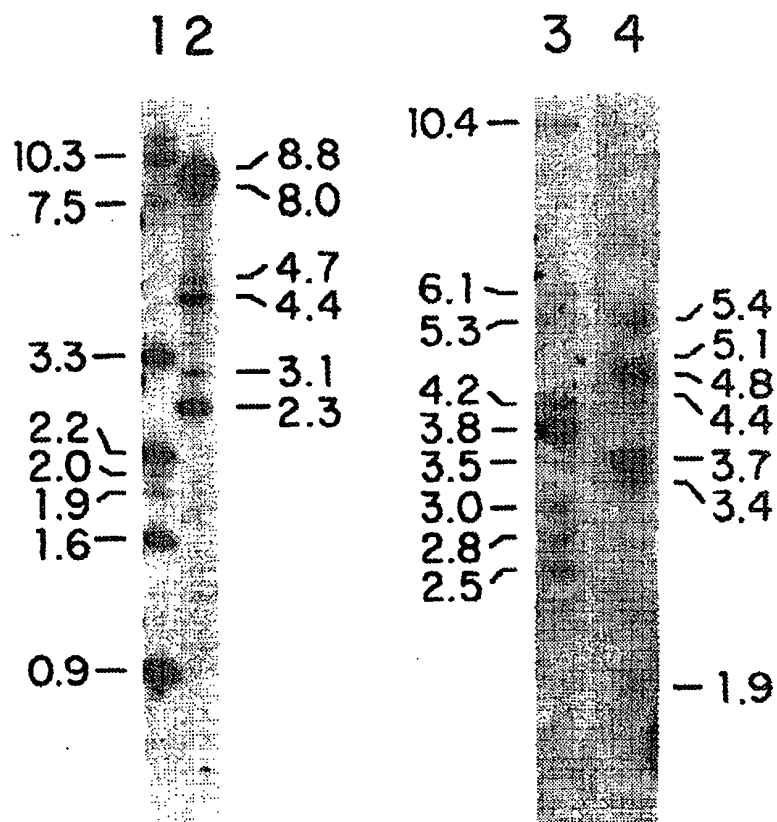
FIG.6
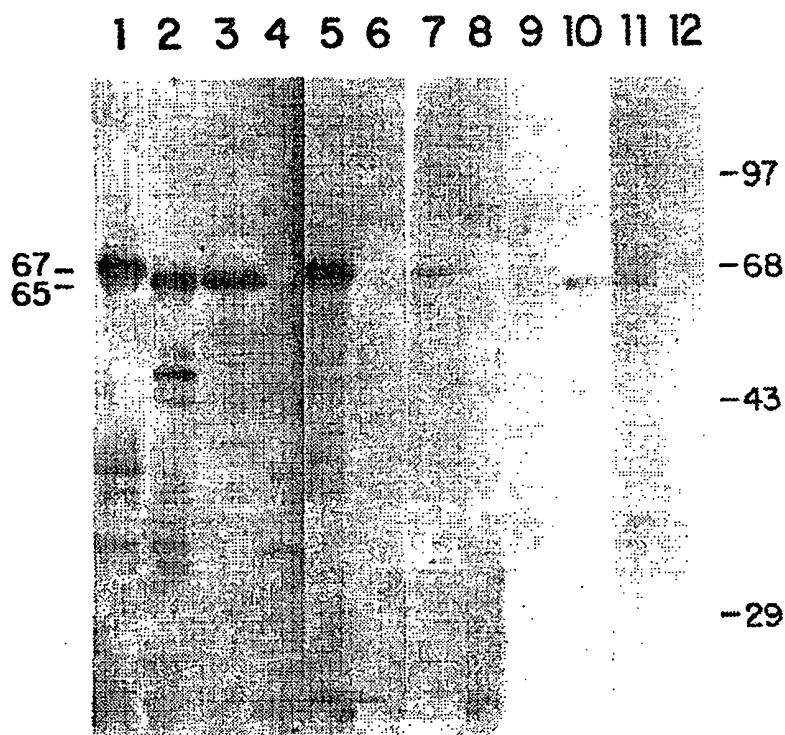
FIG.7
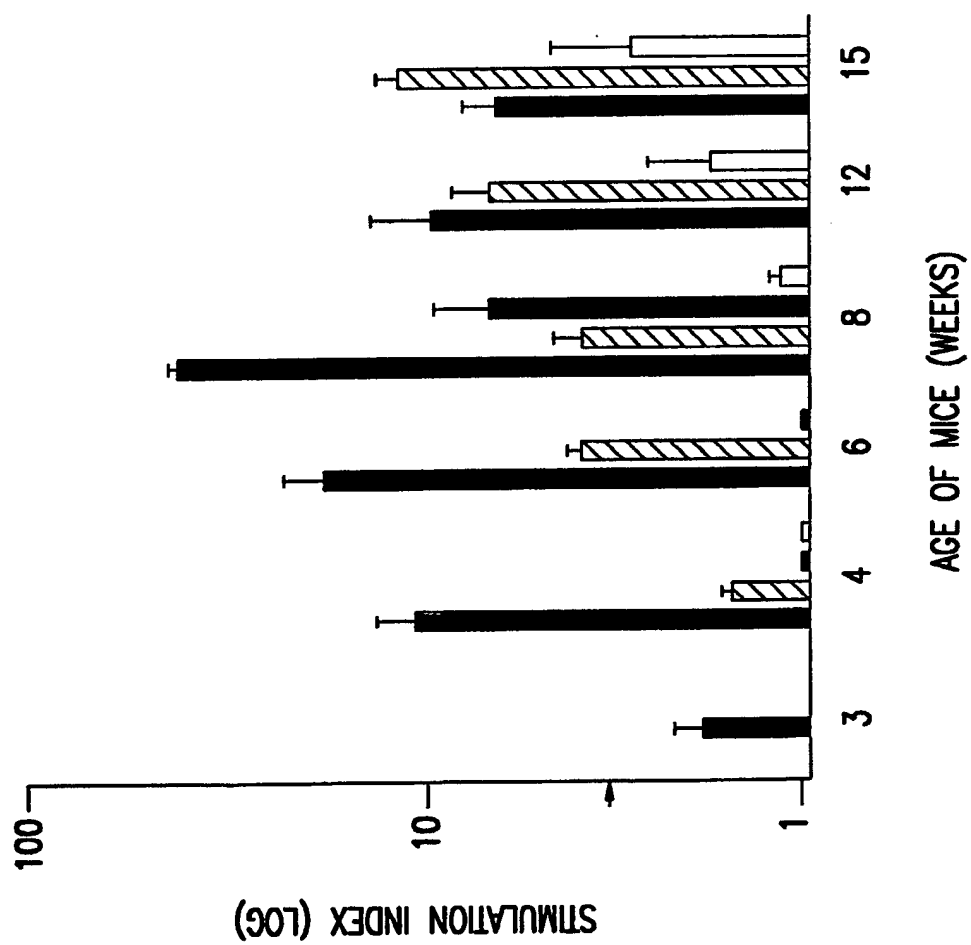
FIG.8
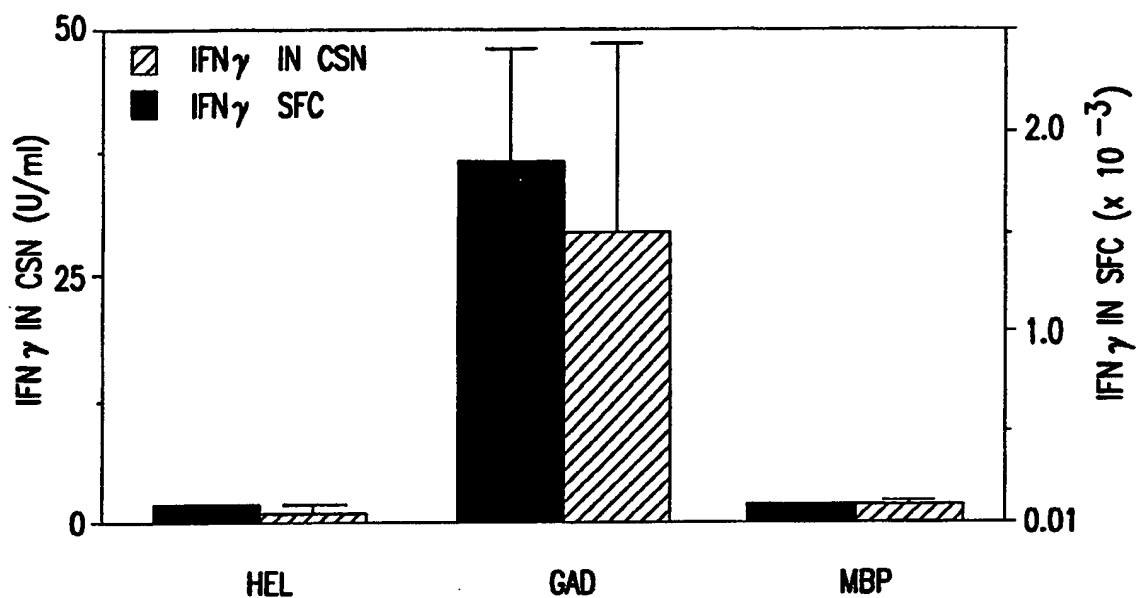
FIG.9A
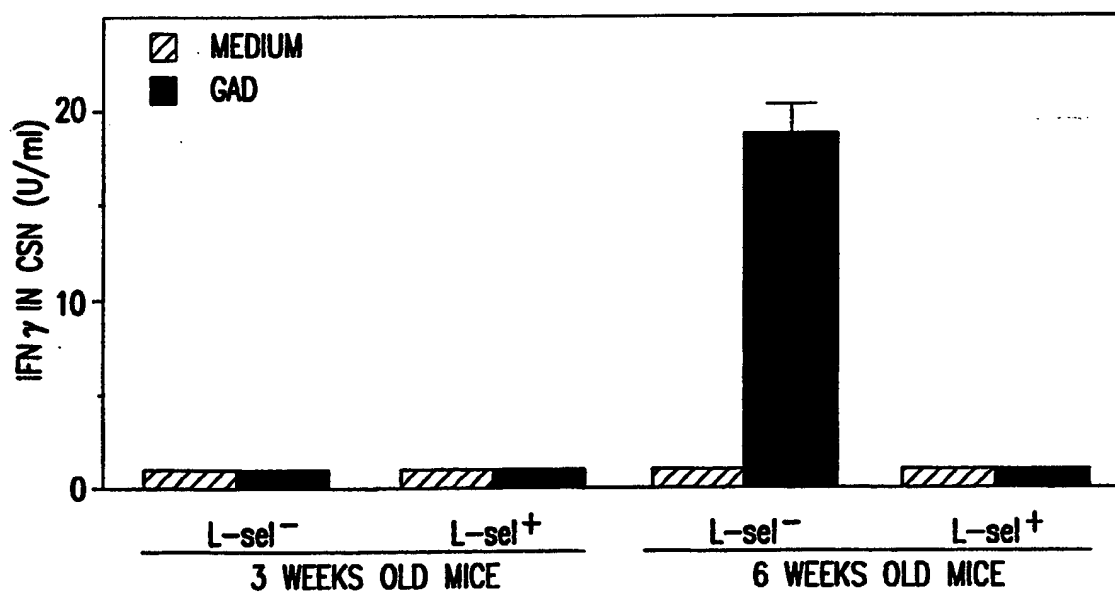
FIG.9B

FIG.10A

FIG.10B

FIG.10C

o = MICE RECEIVING GAD IN IFA

● = MICE RECEIVING CONTROL β –GAL IN IFA

FIG.11